



Discovery of HDL Subspecies and Investigation of Their Associations With Insulin Sensitivity and Early Carotid Atherosclerosis

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**Discovery of HDL subspecies and investigation of their associations with
insulin sensitivity and early carotid atherosclerosis**

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A Dissertation Submitted to the Faculty of
The Harvard T.H. Chan School of Public Health
in Partial Fulfillment of the Requirements
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Harvard University
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**Discovery of HDL subspecies and investigation of their associations with
insulin sensitivity and early carotid atherosclerosis**

Abstract

Recent findings from genome-wide association studies and pharmaceutical trials of CETP inhibitors suggest that HDL cholesterol might not fully capture the anti-atherogenic functions of HDL. The protein component of HDL might be more informative in explaining HDL functions because proteins have characteristics that can modify the biological properties of HDL.

In Chapter 1, I examined whether some proteins located in the HDL size or density range by proteomics studies were present in distinct apoA-I HDL subspecies that could be identified and quantified by a novel sandwich ELISA. Known HDL subspecies containing apoA-II, apoC-III and apoE comprised 70%, 6% and 10%, respectively, and found that novel HDL subspecies containing apoC-I, apoJ, alpha-1 antitrypsin, plasminogen, and apoC-II comprised 14%, 7%, 5%, 3% and 1%, respectively, of the total apoA-I in HDL.

HDL that contains apoC-III has been associated with a higher prevalence of obesity and an elevated risk of CHD, opposite to what has been seen for HDL that does not contain apoC-III. To

further our knowledge on apoC-III-based HDL subspecies, I investigated their associations with insulin sensitivity measured by oral glucose tolerance test (OGTT) in Chapter 2 and early stages of carotid atherosclerosis measured as carotid intima-media thickness (cIMT) in Chapter 3. Using the novel sandwich ELISA established in Chapter 1, I measured the concentrations of apoA-I in HDL with and without apoC-III in the European multi-center “*Relationship between Insulin Sensitivity and Cardiovascular disease*” (RISC) study.

ApoA-I in HDL with and without apoC-III demonstrated significantly opposite associations with both 3-year change in insulin sensitivity and cIMT at baseline. ApoA-I with apoC-III was associated with a decrease in insulin sensitivity and higher cIMT, whereas the concentration of apoA-I without apoC-III was associated with an increase in insulin sensitivity and lower cIMT. The results for apoC-III itself in HDL was consistent with apoA-I with apoC-III. Total apoA-I was null in both cases. These findings suggest that the presence of apoC-III on HDL diminishes and impairs otherwise beneficial effects of HDL on glucose regulation and atheroprotection, and support the potential of HDL apoC-III as a promising target for diabetes and atherosclerosis prevention and treatment.

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Rain Yamamoto

Chapter 1

Identifying and quantifying apolipoprotein A-I High Density Lipoprotein subspecies by a novel sandwich ELISA

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ABSTRACT

Background

HDL is a heterogeneous group of lipoproteins comprised of a variety of proteins with distinct functions and associations with CVD. We hypothesized that some of the proteins located in the HDL size or density range by proteomics studies are present in distinct apoA-I HDL subspecies that can be identified and quantified by a novel sandwich ELISA.

Methods

A microplate coated with antibody against a protein of interest that separates the plasma lipoproteins into bound and unbound fractions. Each fraction is transferred to an anti-apoA-I antibody coated microplate to measure the concentrations of apoA-I with and without the protein.

Findings

We confirmed that known HDL subspecies containing apoA-II, apoC-III and apoE comprised 70%, 6% and 10%, respectively, and found that novel HDL subspecies containing apoC-I, apoJ, alpha-1 antitrypsin, plasminogen, and apoC-II comprised 14%, 7%, 5%, 3% and 1%, respectively, of the total apoA-I in HDL.

Conclusion

We established a novel sandwich ELISA, which demonstrated the existence of eight apoA-I HDL subspecies and showed that they exist in abundance with potential biological importance.

The method is suitable for studying HDL subspecies in relation to dyslipidemia, CVD risk and treatments, and is also applicable to apoB lipoprotein subspecies.

INTRODUCTION

The protective effect of HDL-cholesterol is challenged by pharmaceutical trials of CETP inhibitors and genome-wide association studies, which suggest that HDL-cholesterol may not be a suitable therapeutic target because it may not represent HDL function. The protein composition of HDL may be more relevant than HDL-cholesterol to the risk of CVD because proteins have specific functions that they may impart to HDL. Alaupovic first proposed in 1964 the concept of classifying lipoprotein subspecies on the basis of the protein components of the lipoprotein particles instead of the lipid component and the size (1). Two groups independently demonstrated the concept of HDL subspecies by different immunochemical techniques (2, 3). About two thirds of plasma apoA-I in normolipidemic people is on HDL that also has apoA-II while the rest of apoA-I is on HDL that is free of apoA-II (4-6). Recently we found that HDL exists in subspecies based on the apoC-III and apoE content and that about 6-7% of the total plasma apoA-I in HDL is associated with apoC-III or apoE in people with normal body weight (7). Moreover, in HDL particles associated with apoC-III, there are on average 4 molecules of apoC-III per apoA-I molecule present in the HDL particles. Assuming an average of 4 molecules of apoA-I per HDL particle (8), an HDL that has apoC-III has 16 molecules of it. This is more than enough apoC-III to be present on all HDL particles. A similar calculation can be made for HDL that contains apoE (7). These observations indicate that proteins cluster into certain HDL particles and form subspecies rather than being distributed throughout all HDL particles in plasma.

The classification of HDL into subspecies based on the presence or absence of protein components has clinical significance. The percentage of apoA-I HDL that contains apoC-III or apoE in people with obesity was about 13-14%, which is doubled as compared to people with

normal weight (7). Moreover, HDL without apoC-III was associated with decreased risk of CVD, whereas HDL with apoC-III was associated with increased risk of CVD (9). Of note, the apoC-III concentration in HDL was not significantly associated with CVD. These findings suggest that the concentration of HDL particles with *any* apoC-III may be more relevant with regard to CVD risk than how much apoC-III is contained in HDL.

Over the past decade, HDL proteomics studies have identified many proteins located in HDL (10-25). Davidson has compiled a list of these HDL proteins (<http://homepages.uc.edu/~davidswm/HDLproteome.html>), designating 95 as likely based on the criteria of appearing in 3 separate reports from 3 independent laboratories. The properties of the HDL associated proteins include not only lipid metabolism and transport but also complement regulation, inflammation or acute-phase response, anti-oxidation and anti-thrombosis, hemostasis and protease inhibition.

There is increasing evidence that HDL particles are compositionally heterogeneous. For example, Davidson and colleagues identified the existence of distinct protein clusters in HDL particles and suggested the presence of HDL particle subpopulations each with distinct protein components (15, 16). While it has been shown by immunoaffinity chromatography that apoA-II, apoC-III and apoE define apoA-I-containing HDL subspecies (4, 7), it is yet unknown whether other proteins located in the HDL size or density range by proteomics also constitute apoA-I-containing HDL subspecies.

Therefore, we hypothesized that some of the proteins identified in proteomics studies are present in distinct apoA-I HDL subspecies that can be identified and quantified by a novel sandwich ELISA. Immunoaffinity chromatography has been used as a standard method to characterize lipoprotein particles based on the protein content but it is time-consuming and

impractical for large population studies. In order to determine which proteins constitute subspecies of HDL and to measure the apoA-I concentrations of HDL subspecies, we established a novel sandwich ELISA that is faster and more reproducible than immunoaffinity chromatography. We selected eight proteins with varied functions as shown in Table 1-1. In addition to apoA-II, apoC-III, and apoE, which define known subspecies of apoA-I HDL (4, 7), we also selected apoC-I, apoC-II, apoJ, alpha-1 antitrypsin (A1AT) and plasminogen, to determine whether their association with HDL constitutes new subspecies of apoA-I HDL, and in what concentrations they exist. We confirmed the presence and concentrations of the three known subspecies, and then found that the 5 others were in subspecies using the novel sandwich ELISA. Most of them comprise 5% or more of total apoA-I.

MATERIALS AND METHODS

Blood samples

23 healthy male and female volunteers aged 23 to 64 were recruited from students and staff at Harvard T. H. Chan School of Public Health to create plasma pools. They were asked to fast the night before and up until the blood draw. Blood samples were collected in EDTA-treated tubes, centrifuged, divided into aliquots and stored at -80°C. After blood draw, the plasma samples were immediately anonymized. All volunteers gave informed consent.

Measurement of Lipids and Reference Apolipoprotein Levels in HDL subspecies

The 23 individual samples were removed from cryogenic storage and thawed at room temperature. ApoA-I was measured in whole plasma by standard sandwich ELISA, and cholesterol and triglycerides were measured by enzymatic methods (Infinity Kit, Thermo Fisher

Scientific, Waltham, MA). Based on the content of lipids and apolipoproteins, the 23 individual samples were placed into four groups and those in the same group were combined into one pool: high triglyceride/low HDL cholesterol group (Pool 1), high LDL cholesterol group (Pool 2), high apoA-I group (Pool 3), and low triglyceride/low LDL cholesterol group (Pool 4). After the pools were created, apoA-I, apoB, cholesterol and triglycerides were measured in the four pools.

For assessing the validity of the novel sandwich ELISA against a validated method, we performed immunoaffinity chromatography to obtain reference levels of the four plasma pools for their concentrations of total apoC-III, apoA-I that is associated with apoC-III and apoA-I that is not associated with apoC-III. Each pool was filtered, and the filtered plasma was loaded into 20 ml Econo-Pac columns (Bio-Rad Laboratories, Hercules, CA) packed with anti-apoC-III resin (Sephacrose 4B Resin; Academy Biomedical Company Inc, Houston, TX). Plasma and resin were incubated for 16 hours at 4°C with mixing. The unbound fraction was eluted from the column by gravity followed by washing three times with 4 ml 1x phosphate-buffered saline (PBS). The bound fraction was then eluted from the columns three times with 4 ml 3 mol/l sodium thiocyanate in 1xPBS, followed by washing once with 3 ml PBS and was immediately desalted with the use of concentrators (Spin-X UF Concentrator, Corning). The unbound fraction was measured for the concentration of apoA-I that is not associated with apoC-III, and the bound fraction for the concentration of apoA-I that is associated with apoC-III by standard sandwich ELISA.

For testing the specificity of the novel sandwich ELISA, plasma from three of the 23 individual donors was selected based on the concentration of apoA-I with apoC-III as determined by immunoaffinity chromatography. These three participants were selected to represent low, medium and high levels of apoA-I with apoC-III and were named Participant 1, 2 and 3,

respectively.

Measuring apoA-I concentrations of HDL subspecies using the novel sandwich ELISA

We use apoC-III as an example to describe the assay for the apoA-I concentration that is associated with or without apoC-III (Figure 1-1). A 96-well microplate (Greiner Bio-One MICROLON™ 600, VWR Cat #82050-734) was coated with rabbit anti-human apoC-III antibody (Academy Biomedical, Cat #33A-R1b, 10 µg/ml in 1xPBS) to be used as the first plate (*apoC-III plate*). Two microplates were coated with goat anti-human apoA-I antibody (Academy Biomedical, Cat #11A-G2b, 5 µg/ml in 1xPBS) to be used as the second and third plates (*apoA-I without apoC-III plate* and *apoA-I with apoC-III plate*). The *apoC-III plate* facilitates the fractionation of lipoproteins by apoC-III content and quantifies plasma total apoC-III concentration, the *apoA-I without apoC-III plate* quantifies the apoA-I concentration of HDL that is not associated with apoC-III, and the *apoA-I with apoC-III plate* quantifies the apoA-I concentration of HDL that is associated with apoC-III. The plates were incubated for 1 hour at 37°C and washed three times with 300 µl washing buffer (0.1% Tween 20 in 1xPBS). Then the plates were blocked with blocking buffer (Pierce, Casein in 1xPBS (1% w/v) for 1 hour at 37°C, VWR Cat #PI37528) followed by washing three times with 300 µl washing buffer.

A plasma pool whose apoC-III and apoA-I concentrations had been previously established to be 11.5 mg/dl and 174.0 mg/dl, respectively was chosen to produce a calibration curve. The calibration curve was prepared in dilutions starting at 10,000x and serially 2x further to 640,000x in 1xPBS containing 0.5% BSA. Four pooled plasma samples were prepared at 1:50,000 dilutions. The diluent did not contain Tween 20 so that the lipoprotein particle remained intact

without delipidation. The prepared calibration curve and pooled samples were put in the *apoC-III plate* and incubated overnight at 4°C.

On Day2, the unbound fraction that did not have apoC-III was transferred from the *apoC-III plate* to the *apoA-I without apoC-III plate*. It was mixed well with Tween-containing diluent (1xPBS/2% BSA/0.05% Tween 20). This step was to dilute the unbound fraction to levels that fall within the calibration range. The *apoA-I without apoC-III plate* was then incubated for 1 hour at 37°C. After transferring the unbound fraction to the *apoA-I without apoC-III plate*, the *apoC-III plate* was washed three times gently with 1xPBS and the lipid and protein components of the bound lipoproteins on the *apoC-III plate* were dissociated from anti apoC-III antibody on the plate by Tween-containing diluent incubated for 1 hour at room temperature on the orbital shaker. The dissociated fraction was transferred from the *apoC-III plate* to a third plate, *apoA-I with apoC-III plate* that was coated with anti-apoA-I antibody, and incubated for 2 hours at 37°C.

After these steps, all three plates were washed three times with washing buffer. Horseradish peroxidase (HRP) conjugated anti-apoC-III antibody (Academy Biomedical, Cat #33H-G2b, 1 µg/ml in 1xPBS) was added to the *apoC-III plate* and HRP conjugated anti-apoA-I antibody (Academy Biomedical, Cat #11H-G1b, 1 µg/ml in 1xPBS) was added to the *apoA-I without apoC-III plate* before incubation for 1 hour at 37°C. Following washing three times with washing buffer, o-phenylenediamine (OPD) (Sigma Aldrich, Cat #P9187-50SET) was added to each well on both the *apoC-III and apoA-I without apoC-III plates* to develop color for 1 hour and 20 minutes at room temperature and the absorbance was read at 450 nm. For the *apoA-I with apoC-III plate*, biotinylated anti-apoA-I antibody (Academy Biomedical, Cat #11B-G2b, 1 µg/ml with 1xPBS) was added, incubated for 1 hour at 37°C, washed three times with washing buffer,

followed by addition of avidin peroxidase (Sigma Aldrich, Cat #A7419-2ML, 0.01 µg/ml in 1xPBS), and incubated for 1 hour at 37°C. After washing three times with washing buffer, OPD was added and incubated for 1 hour and 20 minutes before the absorbance was read at 450nm.

Each sample was measured in triplicate. The percentage of apoA-I that has apoC-III was calculated by dividing the concentration of apoA-I with apoC-III (the *apoA-I with apoC-III plate*) by the concentration of total apoA-I (the sum of the *apoA-I without apoC-III* and *apoA-I with apoC-III plates*).

Assays for other HDL subspecies were performed similarly. We also developed an assay for apoB with and without apoC-III by applying the same principle. The sources of coating antibodies and detection antibodies used for each assay are described in Supplementary Table 1-1.

RESULTS

Pooled samples characteristics

Characteristics of lipid and reference apolipoprotein levels of the four pooled samples are shown in Table 1-2.

Sensitivity and specificity

Our modified sandwich ELISA was demonstrated to be very sensitive. The absorbance decreased dose-dependently as the dilutions increased from 10,000x serially 2x up to 640,000x for all the three measures: total apoC-III, apoA-I without apoC-III and apoA-I with apoC-III (Figure 1-2). The lower limit of quantification for ELISA was established at 0.018 µg/dl for apoC-III, 0.026 µg/dl for apoA-I without apoC-III, and 0.159 µg/dl for apoA-I with apoC-III.

To assess the specificity of the newly developed ELISA, plasma samples depleted of apoC-III by immunoaffinity chromatography were measured for content of apoC-III and of apoA-I with and without apoC-III using the modified sandwich ELISA. For comparison, an apoC-III undepleted (unaltered) plasma sample was measured as well. For this experiment, we used three individual plasma samples from Participants 1, 2 and 3, who had low, medium and high levels, respectively, of apoA-I with apoC-III determined by immunoaffinity chromatography. Plasma apoC-III was detected only in the undepleted samples (Figure 1-3A). Both apoC-III depleted and undepleted samples showed absorbance on the *apoA-I without apoC-III* plate (Figure 1-3B). The concentration of apoA-I without apoC-III was 20-25% lower in the depleted sample as compared to the unaltered sample, which is likely due to loss during immunoaffinity chromatography. The plasma depleted of apoC-III showed no absorbance on the *apoA-I with apoC-III* plate, indicating a good specificity, while the undepleted sample showed absorbance in a concentration-dependent manner (Figure 1-3C).

Validity

To evaluate the validity of the assay against a validated method, we compared the concentrations of apoC-III and apoA-I with and without apoC-III obtained from the modified sandwich ELISA with the reference levels determined by immunoaffinity chromatography. The correlations between the values obtained by ELISA and the ones determined by immunoaffinity chromatography were 0.96 for apoC-III, 0.91 for apoA-I without apoC-III, and 0.89 for apoA-I with apoC-III (Figure 1-4A). The mean values obtained by our novel ELISA method compared to the reference values determined by immunoaffinity chromatography are shown in Figure 1-4B.

Reproducibility

Reproducibility within an assay (intra-assay CV%) and between assays (inter-assay CV%) was compared between the modified sandwich ELISA and immunoaffinity chromatography (Table 1-3).

The modified sandwich ELISA had lower intra-assay CV% than immunoaffinity chromatography. The inter-assay CV% from ELISA was substantially lower than that from immunoaffinity chromatography, particularly for apoC-III and apoA-I with apoC-III. Thus, ELISA had consistently better reproducibility both within an assay and between assays than immunoaffinity chromatography.

Other proteins associated with apoA-I HDL

We quantified the concentrations of eight HDL subspecies each containing either apoA-II, apoC-III, apoE, apoC-I, apoC-II, apoJ, A1AT, or plasminogen. The mean values of the four pools and the percentage of apoA-I HDL that was associated with each protein are shown in Figure 1-5A. Out of the total apoA-I concentration of 147 mg/dl, the majority of ApoA-I was found to be associated with apoA-II; 70% of apoA-I (mean \pm SD: 102 ± 26 mg/dl) had apoA-II, whereas the remaining 30% of apoA-I was apoA-II free. 10% of apoA-I (14 ± 2 mg/dl) was associated with apoE, and 6% (9 ± 0.5 mg/dl) with apoC-III. These observations were consistent with the previously reported values (4, 7). Among the novel HDL subspecies, the most abundant one was apoC-I-containing HDL, with 14% of apoA-I in association with apoC-I (20 ± 5 mg/dl). HDL subspecies containing apoJ, A1AT, plasminogen and apoC-II comprised 7% (10 ± 0.5

mg/dl), 5% (7 ± 0.7 mg/dl), 3% (4 ± 0.2 mg/dl) and 1% (1.5 ± 0.3 mg/dl) of the total apoA-I in HDL, respectively.

ApoB lipoproteins with apoC-III

We found that 7% of apoB (6 ± 0.8 mg/dl) was associated with apoC-III (Figure 1-5B), which also agrees with our previous finding using immunoaffinity chromatography (26).

DISCUSSION

To examine whether proteins identified to be located in HDL size or density range by proteomics studies also constitute apoA-I HDL subspecies, we developed a novel sandwich ELISA that can measure the concentrations of apoA-I HDL subspecies with or without a protein of interest. We confirmed that the three known HDL subspecies containing apoA-II, apoC-III and apoE comprised 70%, 6% and 10% of total apoA-I HDL, respectively. In addition, we identified and quantified five new HDL subspecies containing apoC-I, apoJ, A1AT, plasminogen and apoC-II that comprised 14%, 7%, 5%, 3% and 1% of apoA-I HDL, respectively. This finding is important because some of the novel HDL subspecies might also differ in their association with diseases such as CHD and diabetes and therefore serve as valuable predictors. We also showed that our modified sandwich ELISA is also applicable to apoB lipoprotein subspecies.

The eight proteins investigated have unique properties that may confer specific functions to HDL. HDL proteomics studies have compared protein profiles of CAD patients with those of healthy people and found that some proteins were enriched in HDL of patients with CAD (14, 17, 25). The findings from these studies, however, were not consistent with regard to

which HDL proteins were enriched in CAD patients. Moreover, these studies were limited because of the small sample size and the cross-sectional design. In two large prospective cohort studies, the Nurses Health study and Health Professional Follow-up study, we found that HDL that does not contain apoC-III was associated with decreased CHD risk but HDL that contains apoC-III was associated with increased CHD risk (9). HDL seems to provide a platform or act as a vehicle for such proteins to exert their effect. In mice, treatment with A1AT-enriched HDL resulted in greater protection against elastase-induced pulmonary emphysema than treatment with HDL or A1AT alone, demonstrating that vectorization of A1AT by HDL provides some sort of directionality towards sites containing free elastase and represents a better means of delivering A1AT (27). Furthermore, supplementation of HDL with purified clusterin (apoJ) increased its endothelial anti-apoptotic effects, whereas clusterin alone did not have such effects (25). Some of the proteins may also influence kinetics of HDL. For example, apoA-II-containing apoA-I HDL have a slower turnover rate than non-apoA-II-containing apoA-I HDL in normolipidemic people (6), which was reversed in patients with LCAT deficiency (5). In abetalipoproteinemia, apoE containing HDL is cleared more rapidly from the circulation than HDL that does not have apoE (28).

Immunoaffinity chromatography has played an important role thus far for the speciation of lipoproteins such as apoE-containing apoB lipoproteins and apoC-III-containing apoA-I lipoproteins. This method, however, has a number of disadvantages; the assay is time-consuming and costly, and has fairly high inter- and intra-assay CV%. We demonstrated in this paper that our modified sandwich ELISA could attenuate these disadvantages. First, the novel ELISA is faster because the entire protocol is completed in one day after overnight incubation of plasma samples, while the immunoaffinity chromatography method requires an additional day. Second, the

modified sandwich ELISA can process a larger number of samples per assay because of simpler procedures. Unlike immunoaffinity chromatography, the modified sandwich ELISA does not require elution of bound lipoproteins from a column and concentration of samples. Third, it is substantially cheaper because this method requires much less antibody. Finally, we confirmed the validity of the modified sandwich ELISA against immunoaffinity chromatography and showed that our modified sandwich ELISA has improved reproducibility both within an assay and between assays. The advantages of the modified sandwich ELISA make studies of the associations between HDL subspecies and disease feasible, and allow risk prediction in large population studies.

Previous efforts have sought to establish a convenient ELISA that measure lipoprotein subspecies. Fruchart and colleagues developed a sandwich ELISA, where they capture apoC-III, for example, by a coating antibody, add apoB detection antibody conjugated to peroxidase, and develop by OPD, without delipidating the lipoprotein particle (29). This seemingly simple assay, however, has not been used subsequently to our knowledge. We tried to apply their ELISA to measure apoA-I with and without apoC-III but this method did not work for our purpose because it produced only weak signals. One possible explanation is that the epitopes of the apoA-I protein may be hidden by apoC-III, which exists in association with the HDL particle far more abundantly than apoA-I. We found that an average of four apoC-III molecules per apoA-I molecule are present in the HDL subspecies that has apoC-III (7). Because most HDL have four molecules of apoA-I (8), approximately 16 molecules of apoC-III could reside on apoC-III containing HDL.

Our modified sandwich ELISA method has limitations, however. It cannot measure the concentration of HDL associated with multiple proteins simultaneously, while it is likely that some HDL particles contain more than one protein in addition to apoA-I. High throughput

methods such as mass spectrometry allow identification of many proteins in HDL all at once. However, mass spectrometry can only measure the concentrations of HDL proteins themselves, which may not be informative in the CVD risk prediction based on our previous finding that it was the concentration of apoA-I associated with apoC-III that predicted CVD risk, more so than the concentration of apoC-III contained in HDL (9). Another limitation is that the modified sandwich ELISA requires three ELISA plates in total, but it provides three independent measurements: plasma total apoC-III, apoA-I in HDL with apoC-III, and apoA-I in HDL without apoC-III.

In conclusion, we established a novel sandwich ELISA, which confirmed the existence of eight apoA-I HDL subspecies investigated and showed that they exist in abundance with potential biological importance. The method is suitable for studying HDL and apoB lipoprotein subspecies in relation to dyslipidemia, CVD risk and treatments in large population studies. Our novel ELISA method may also enable a diagnostic test with an improved CVD risk predictability in the future.

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Table 1-1. Eight proteins selected for discovery and characterization of apoA-I HDL subspecies

	Reference plasma levels (mg/dl)	Molecular weight (KDa)	Functions
ApoA-II	45	17	The second major HDL apolipoprotein. Lipid metabolism and transport
ApoC-I	11	6.6	An LCAT activator and CETP inhibitor when associated with HDL. Inhibitor of LPL and hinders apoE mediated uptake of lipoproteins by the liver
ApoC-II	7	8.8	Activates LPL
ApoC-III	14	8.8	Hinders apoE mediated uptake of lipoproteins by the liver. Both LDL and HDL associated with apoC-III predict increased CVD risk
ApoE	9	34	Mediates uptake of lipoproteins by the liver
ApoJ	5	70	A complement inhibitor. Lipid transport. Apoptosis inhibitor
Plasminogen	13	83	Precursor of plasmin, which lyses fibrin clots to fibrin degradation products, Resolves clotting
Alpha1-antitrypsin	175	52	Serine proteinases inhibitor, Stabilizes plaque

Table 1- 2. Characteristics of four plasma pools of healthy participants

	Pool 1	Pool 2	Pool 3	Pool 4
	(high TG / low HDL chol)	(high LDL chol)	(high total apoA-I)	(low TG / low LDL chol)
Age, median (range)	32 (27-57)	44 (29-57)	30 (27-64)	29 (23-35)
Gender				
Male	2	2	0	3
Female	3	2	5	6
Total Cholesterol (mg/dl)	178	224	174	157
LDL Cholesterol (mg/dl)	113	157	102	95
HDL Cholesterol (mg/dl)	36	45	45	49
TG (mg/dl)	139	116	116	67
Total apoA-I (mg/dl)	123	147	174	142
ApoA-I without apoC-III (mg/dl)	116	138	163	134
ApoA-I with apoC-III (mg/dl)	7.0	9.2	11.4	8.0
Total apoB (mg/dl)	92	82	68	59

Table 1-3. Intra-assay and inter-assay reproducibility

	Intra-assay CV%			Inter-assay CV%		
	ApoC-III	ApoA-I without apoC-III	ApoA-I with apoC-III	ApoC-III	ApoA-I without apoC-III	ApoA-I with apoC-III
Immunoaffinity Chromatography	6.6	7.1	11.6	29.3	15.7	19.9
ELISA	4.7	5.2	8.4	8.6	13.6	8.2

Values are shown as the mean of the four pools.

Inter-assay CV% was calculated from three independent assays.

Intra-assay CV% was calculated from triplicates within each assay and the means of three assays are shown.

Supplementary Table 1-1. Antibodies and optimal conditions for the modified sandwich ELISA

	Protein	Coating Antibody	Detection Antibody for the first plate ^a	Detection Antibody for the second plate ^b	Detection Antibody for the third plate ^c
ApoA-I HDL proteins	ApoA-II	Goat Anti-Human ApoA-II, Academy Biomedical (#12A-G1b, 5 µg/ml)	HRP-Goat Anti-Human ApoA-II, Academy Biomedical (#12H-G2b, 1 µg/ml)	HRP-Goat Anti-Human ApoA-I, Academy Biomedical (#11H-G1b, 1 µg/ml)	Biotin-Goat Anti-Human ApoA-I, Academy Biomedical (#11B-G2b, 1 µg/ml)
	ApoC-I	Rabbit Anti-Human ApoC-I, Academy Biomedical (#31A-R1b, 5 µg/ml)	HRP-Goat Anti-Human ApoC-I, Academy Biomedical (#31H-G1b, 1 µg/ml)		
	ApoC-II	Rabbit Anti-Human ApoC-II, Academy Biomedical (#32A-R1b, 10 µg/ml)	HRP-Goat Anti-Human ApoC-II, Academy Biomedical (#32H-G4b, 1 µg/ml)		
	ApoC-III	Rabbit Anti-Human ApoC-III, Academy Biomedical (#33A-R1b, 10 µg/ml)	HRP-Goat Anti-Human ApoC-III, Academy Biomedical (#33H-G2b, 1 µg/ml)		
	ApoE	Goat Anti-Human ApoE, Academy Biomedical (#50H-G1b, 10 µg/ml)	HRP-Goat Anti-Human ApoE, Academy Biomedical (#50S-G1b, 1 µg/ml)		
	ApoJ	Goat Anti-Human ApoJ, Millipore (#AB825, 5 µg/ml)	Goat Anti-Human ApoJ, Millipore (#AB825 ^d , 1 µg/ml)		
	A1AT	Rabbit Anti-Human A1AT, NOVUS (#NBP1-78098, 5 µg/ml)	Rabbit Anti-Human A1AT, NOVUS (#NBP1-78098 ^d , 1 µg/ml)		
	Plasminogen	Goat Anti-Human Plasminogen, Academy Biomedical (#PG60A-G1b, 10 µg/ml)	HRP-Goat Anti-Human Plasminogen, Academy Biomedical (#PG60H-G1a, 1 µg/ml)		

ApoB protein	ApoC-III	Rabbit Anti-Human ApoC-III, Academy Biomedical (#33A-R1b, 10 µg/ml)	HRP-Goat Anti- Human ApoC-III, Academy Biomedical (#33H-G2b, 1 µg/ml)	HRP-Goat Anti- Human ApoB- 100/48, Academy Biomedical (#20H-G1b, 1 µg/ml)	Biotin-Goat Anti-Human ApoB-100/48, Academy Biomedical (#20B-G1b, 1 µg/ml)
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^a: Plate that measures the concentration of the protein of interest

^b: Plate that measures the concentration of apoA-I or apoB that is not associated with the protein of interest

^c: Plate that measures the concentration of apoA-I or apoB that is associated with the protein of interest

^d: HRP was conjugated to the same antibody as the coating antibody (EZ-link Plus Activated Peroxidase, Thermo SCIENTIFIC, #31489).

Figure 1-1. The novel sandwich ELISA procedure, using apoA-I with and without apoC-III as an example

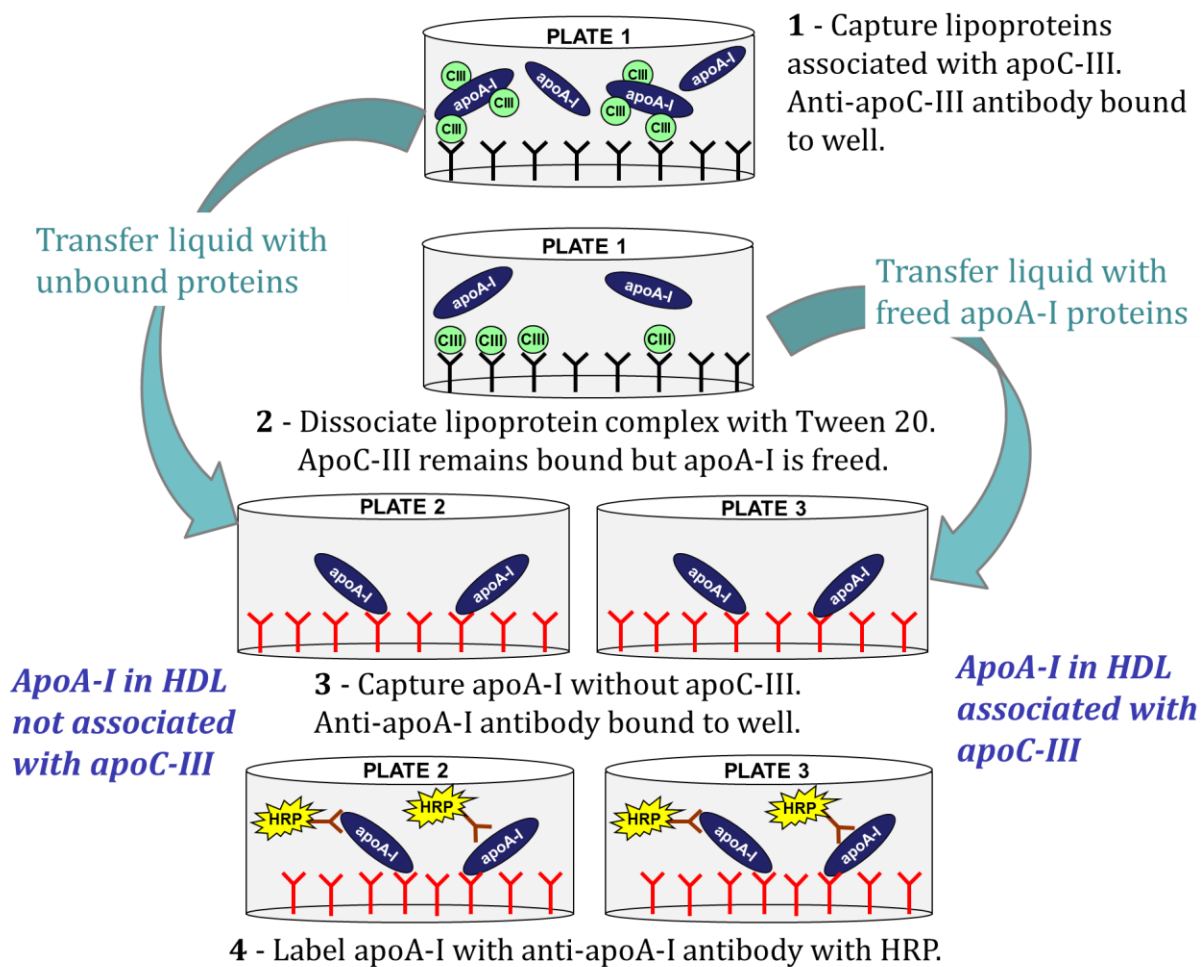
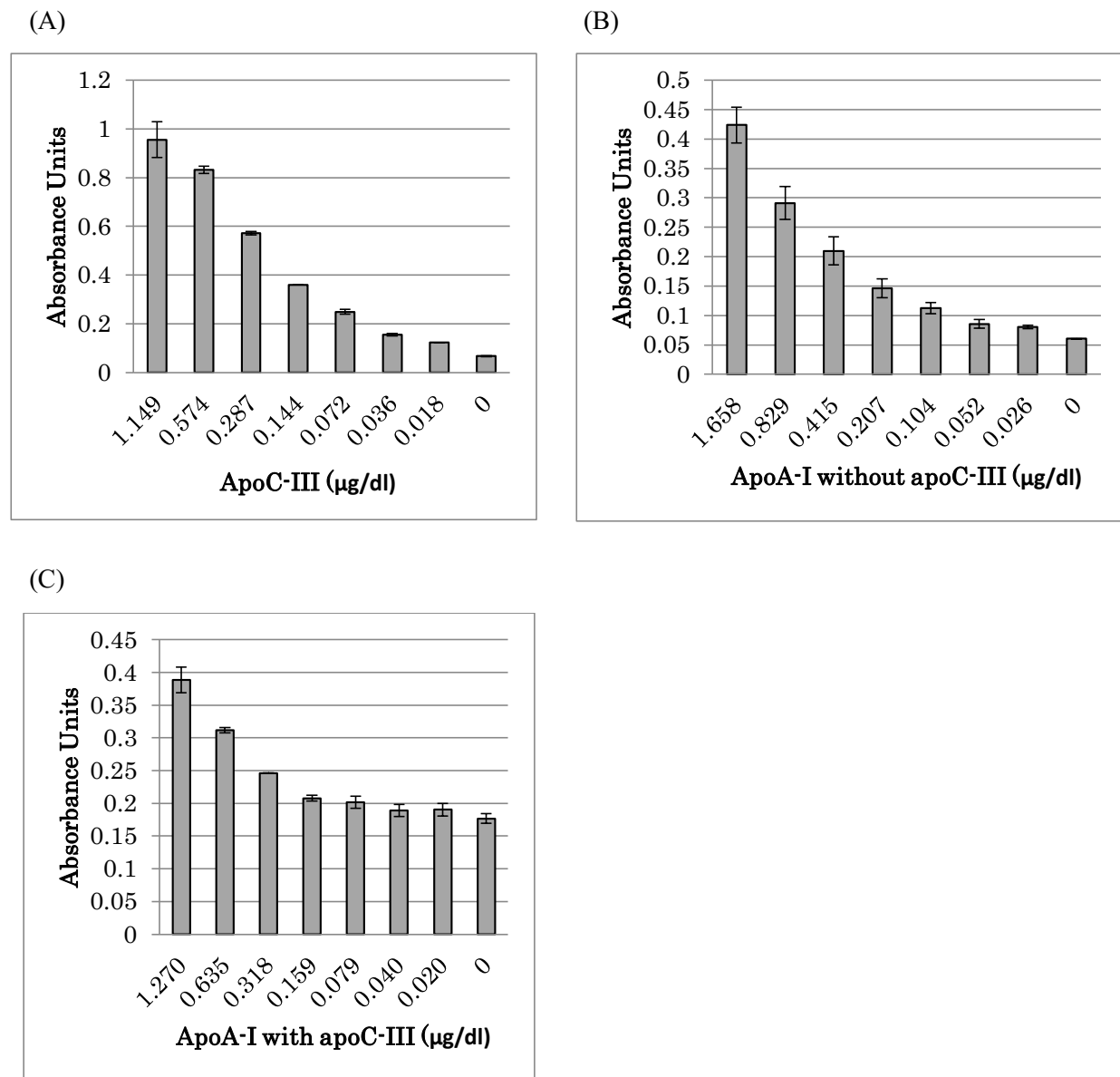
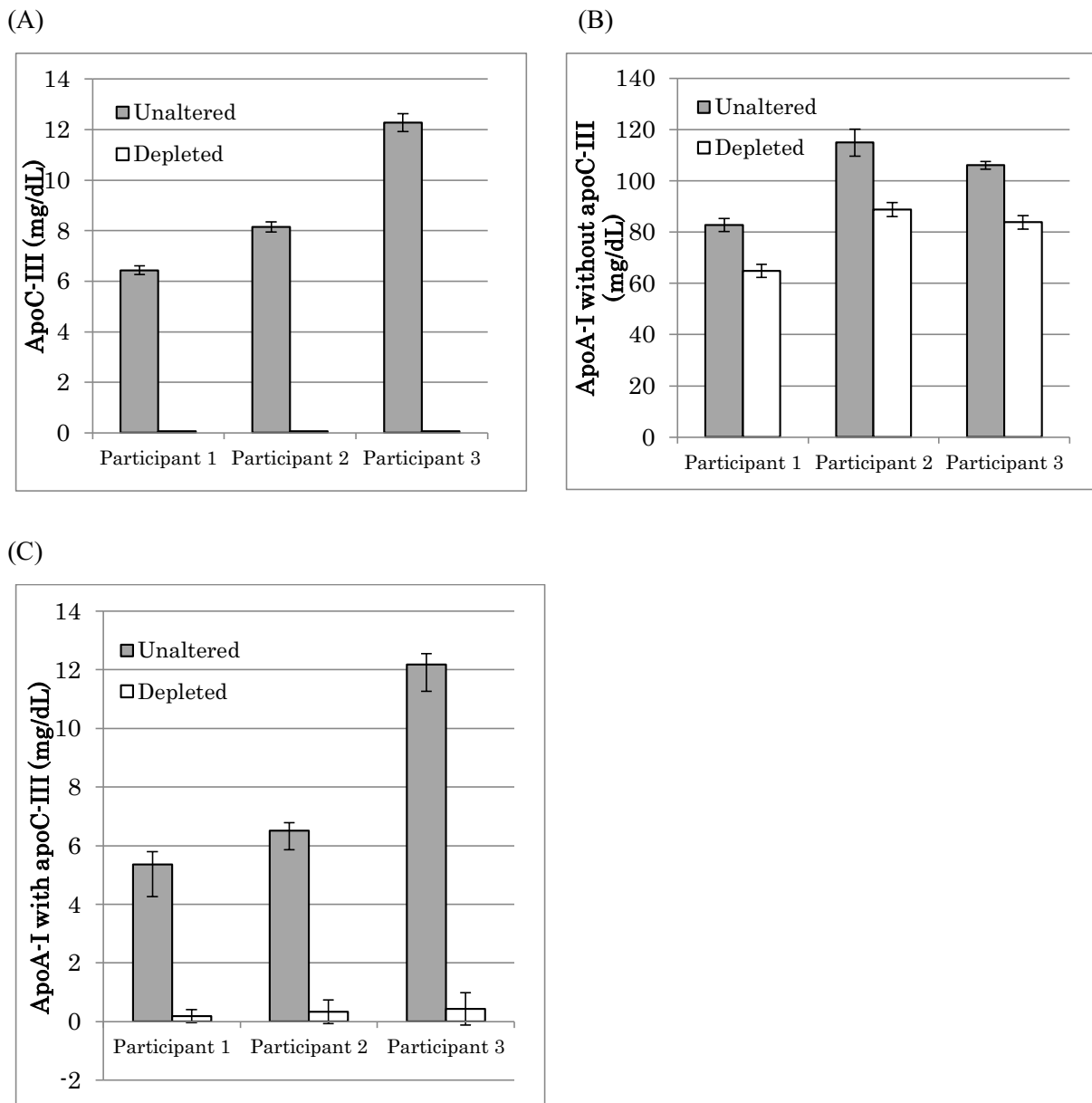


Figure 1-2. Absorbance values of a serially diluted plasma sample



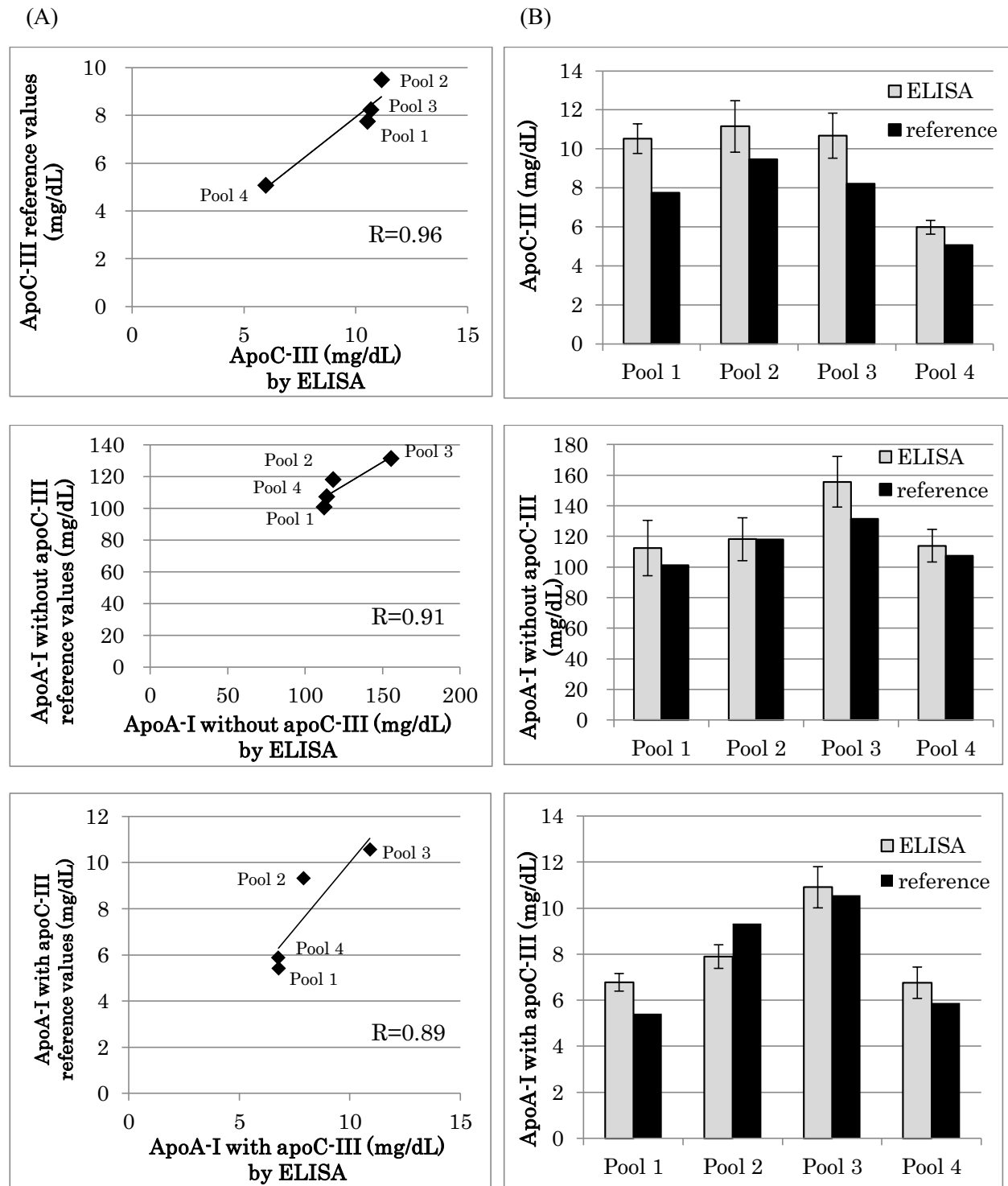
Data are the mean of 2 separate assays (each measured in triplicate) with bars indicating \pm se. The highest concentration corresponds to 1:10,000 dilution and the sample was diluted serially 2x up to 1:640,000. The absorbance decreased dose-dependently as the dilutions increased for (a) total apoC-III, (b) apoA-I without apoC-III and (c) apoA-I with apoC-III.

Figure 1-3. Comparison of unaltered plasma samples to those same samples depleted of apoC-III-containing lipoproteins



Plasma samples with low (Participant 1), medium (Participant 2) and high (Participant 3) levels of apoA-I with apoC-III were used. Data are the mean of 2 separate assays (each measured in triplicate) with bars indicating \pm se. (a) Plasma apoC-III was detected only in unaltered samples. (b) ApoA-I without apoC-III was detected both in unaltered and altered samples. (c) ApoA-I with apoC-III was detected only in unaltered samples.

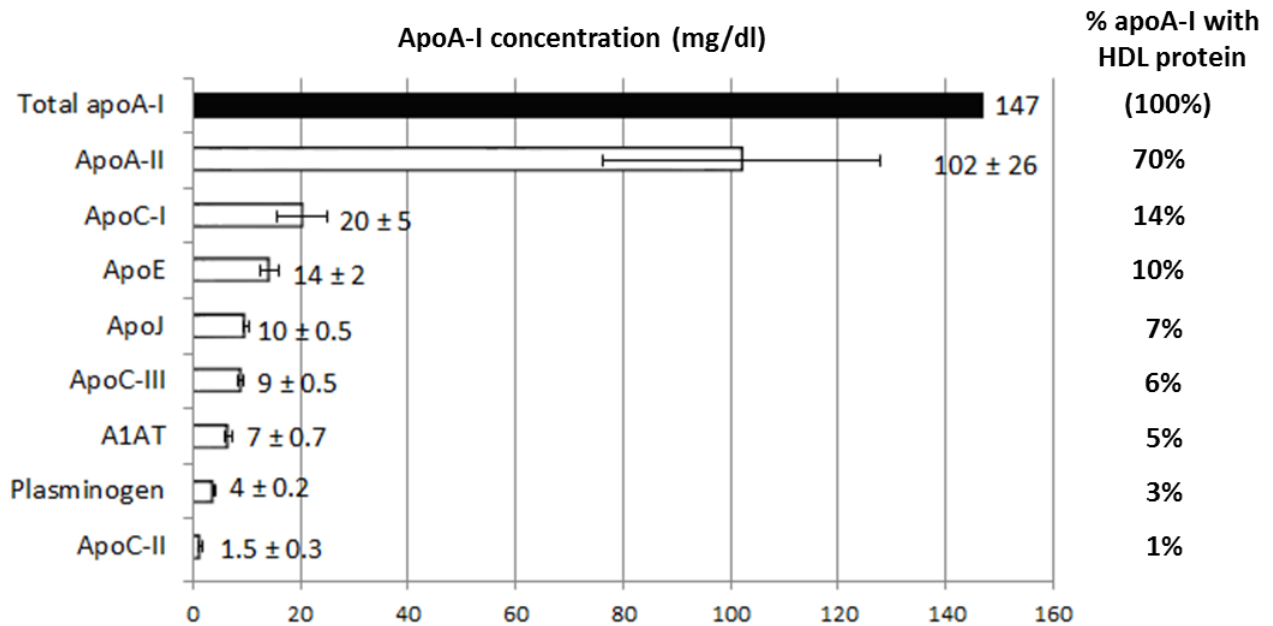
Figure 1-4. Comparison of the values for the four plasma pools (Pools 1-4) obtained from the modified sandwich ELISA with reference levels determined by immunoaffinity chromatography



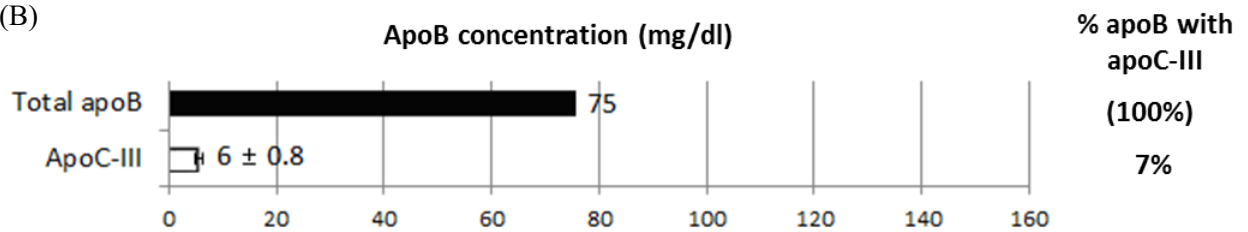
Data are the mean of five separate assays (each measured in triplicate) with bars indicating \pm sd. (a) The correlations between the two methods were 0.96, 0.91 and 0.89 for apoC-III, apoA-I without apoC-III and apoA-I with apoC-III, respectively. (b) The mean absolute deviations of the ELISA values from immunoaffinity chromatography were 25%, 9% and 15% for apoC-III, apoA-I without apoC-III and apoA-I with apoC-III, respectively.

Figure 1-5. The concentrations of apoA-I associated with HDL proteins (a) and the concentration of apoB associated with apoC-III (b)

(A)



(B)



Each data point is the average of the four pools. On the right is the percentage of apoA-I or apoB associated with each HDL protein, calculated as $\frac{\text{ApoA-I with HDL protein}}{\text{Total apoA-I}}$ or $\frac{\text{ApoB with apoC-III}}{\text{Total apoB}}$.

Chapter 2

Apolipoprotein C-III diminishes the beneficial association between HDL and insulin sensitivity: a multi-center cohort study

Rain Yamamoto, Frank M. Sacks, Bernard Rosner, Sarah Aroner, Frank Hu, Andrea Natali, Simona Baldi, Beverley Balkau, Majken K. Jensen

ABSTRACT

Background

Recent findings suggest that HDL directly exerts beneficial effects on the regulation of glucose metabolism. Previously we found that a subspecies of plasma HDL, comprising 6-7% of HDL-apoA-I, has apolipoprotein C-III (apoC-III), a protein known for its proinflammatory properties. We hypothesized that the antidiabetic properties of HDL are attributed only to HDL that does not have apoC-III and that if apoC-III is present on HDL, apoC-III would diminish the beneficial effects of HDL on insulin synthesis and secretion.

Methods

We investigated the associations between the apoA-I concentrations of HDL subspecies with and without apoC-III and insulin sensitivity over 3 years among 864 participants from the European multi-center “*Relationship between Insulin Sensitivity and Cardiovascular disease*” (RISC) study. The concentrations of apoC-III-based HDL subspecies were measured at baseline (2002) by a sandwich ELISA. Insulin sensitivity was measured by oral glucose tolerance test (OGTT) at baseline and year 3.

Findings

ApoA-I in HDL with and without apoC-III demonstrated significantly opposite associations with 3-year change in insulin sensitivity (p -heterogeneity=0.004). The highest quintile of apoA-I in HDL with apoC-III was associated with a 1.2% reduction in insulin sensitivity (p -trend=0.02), while the highest quintile of apoA-I without apoC-III was associated with a 1.3% improvement

(p -trend=0.01), compared to the lowest quintile. Total apoA-I was not associated with 3-year change in insulin sensitivity ($p=0.3$). The concentration of apoC-III contained in HDL was associated with a decrease in insulin sensitivity (-1.3% per 1 SD higher level; $p=0.002$) even more strongly than plasma total apoC-III (-0.6% per 1 SD higher level; $p=0.11$).

Interpretation

Both the concentration of an HDL subspecies that has apoC-III and the concentration of apoC-III itself in HDL appeared to adversely affect the beneficial properties of HDL on the insulin response to glucose. Our results support the potential of HDL apoC-III as a promising target for diabetes prevention and treatment.

INTRODUCTION

HDL is a heterogeneous group of lipoprotein particles. We recently found that HDL exists in subspecies based on the presence of apolipoprotein (apo) C-III, a small protein known for its proinflammatory properties. In healthy adults, apoC-III is present on about 6-7% of HDL, as measured by the plasma total apoA-I, the main HDL apolipoprotein (1), whereas the remaining HDL is free of apoC-III. The classification of HDL into subspecies based on the presence or absence of apoC-III may have important implications for disease risk, as apoC-III-based HDL subspecies differ in their metabolic properties and downstream biologic interactions; HDL that contains apoC-III has been associated with a higher prevalence of obesity and an elevated risk of coronary heart disease, opposite to what has been seen for total HDL or HDL that does not contain apoC-III (1, 2).

Recent evidence suggests that apoC-III-based HDL subspecies might similarly relate to the development of insulin resistance and diabetes. Several studies have suggested that HDL and apoA-I exert direct beneficial effects on the regulation of glucose metabolism through multiple mechanisms (3-5). However, we speculate that these antidiabetic effects of HDL are attributed only to HDL without apoC-III, and if apoC-III is present on HDL, apoC-III would diminish the beneficial effect of HDL on glycemic control. In cross-sectional analyses in the Nurses' Health Study and Health Professionals Follow-Up Study, prevalent diabetes was associated with higher levels of HDL with apoC-III and lower levels of HDL without apoC-III (2).

Indeed, apoC-III has a role in the pathogenesis of insulin resistance and diabetes. Transgenic mice with human apoC-III overexpression have been shown to develop hepatic triglycerides accumulation and diet-induced insulin resistance (6). Mechanistic studies in cells and animal models have demonstrated that apoC-III increases cytokine expression in cultured monocytes

and endothelial cells (7-9) and pancreatic β -cell apoptosis via hyperactivation of β -cell CaV channels (10, 11). These mechanisms link apoC-III to inflammation, pancreatic β -cell function and insulin resistance. Elevated plasma apoC-III concentration is a feature of dyslipidemia in obesity and observed in both type 1 and 2 diabetes (11-13). Several cross-sectional studies (14-16) and one prospective cohort study (17) showed that high levels of apoC-III were associated with diabetes or metabolic syndrome. Further, a group of New York Ashkenazi Jews having genetically reduced plasma apoC-III concentration maintained greater insulin sensitivity with age and reached exceptional longevity (18).

Therefore, we hypothesized that only HDL without apoC-III would be beneficially associated with insulin sensitivity, whereas HDL with apoC-III would be associated with impaired insulin sensitivity. We also studied the concentration of total apoC-III as well as the concentration of apoC-III itself that is contained in HDL. In addition, we examined apoB-containing lipoproteins, VLDL and LDL, with and without apoC-III.

MATERIALS AND METHODS

Study design and participants

RISC is a European multi-center, prospective cohort study with a standardized protocol and a centralized evaluation of measures (19). Healthy men and women ages 30-60 (n=1,513) were recruited between June 2002 and July 2004 from the local population at 19 centers in 14 European countries (Austria, Denmark, Finland, France, Germany, Greece, The Netherlands, Ireland, Italy, Sweden, Spain, Switzerland, United Kingdom and Serbia and Montenegro). Follow-up examinations were performed after 3 years. Exclusion criteria were treatment for any

chronic disease, pregnancy, any cardiovascular event, weight change of ≥ 5 kg in the last 3 months, cancer in the last 5 years, and renal or liver failure, recent major surgery, seizure disorder or epilepsy, arterial blood pressure $\geq 140/90$ mmHg, fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dl), 2-hour plasma glucose (on a standard 75-g OGTT) ≥ 11.0 mmol/L (200 mg/dl), total serum cholesterol ≥ 7.8 mmol/L (300 mg/dl), serum triglycerides ≥ 4.6 mmol/L (400 mg/dl), electrocardiogram abnormalities or acute myocardial ischemia injury or pericarditis. Out of 1,017 eligible subjects whose apolipoprotein levels were initially measured by the RISC investigators, baseline plasma samples of 987 subjects, which had enough plasma left for our experiment, were sent to our laboratory. Each recruiting center obtained approval from local ethics committee.

Exposure assessment

Information on basic characteristics of the participants including demographics (age, sex) and lifestyle (smoking, alcohol consumption, physical activity based on the International Physical Activity Questionnaire (IPAQ) score) were collected by interviews and questionnaires. Anthropometric measures and lipid levels (LDL-, HDL-, total-cholesterol and triglycerides) were also measured (19).

We used a novel sandwich ELISA that we recently developed to measure the concentrations of total apoC-III, apoA-I in HDL without apoC-III, apoA-I in HDL with apoC-III; and the concentrations of apoB in lipoproteins with and without apoC-III (Yamamoto et al 2015, submitting). Briefly, a 96-well microplate coated with anti-human apoC-III antibody captured plasma lipoproteins that contained apoC-III. The unbound fraction was transferred to another plate coated with anti-human apoA-I antibody to measure the concentration of apoA-I that was

not associated with apoC-III. The lipoproteins bound to the anti-apoC-III plate were dissociated with Tween-20, and then transferred to a plate coated with anti-human apoA-I antibody to measure the concentration of apoA-I that contained apoC-III. Total apoA-I was calculated as the sum of the concentrations of apoA-I with and without apoC-III. The concentrations of apoB with and without apoC-III were measured by applying the same principle for the apoA-I subspecies.

For the measurement of the apoC-III concentration in HDL, whole plasma was precipitated with dextran sulfate and magnesium chloride to remove apoB-containing lipoproteins, and the apoC-III concentration in the remaining fraction was measured by standard sandwich ELISA. The apoC-III concentration in HDL is different from the apoA-I concentration of HDL with apoC-III; the former represents the concentration of apoC-III itself contained in HDL whereas the latter represents the concentration of apoA-I in HDL with *any* amount of apoC-III. The apoC-III to apoA-I molar ratio, which may be interpreted as the density of apoC-III on apoC-III-containing HDL, was calculated as the number of apoC-III molecules per apoA-I molecule on HDL particles containing apoC-III. To correct apolipoprotein measurements for moderate variability by batch, values of each apolipoprotein exposure were recalibrated to represent the average distribution across batches using methods developed by Rosner et al (20). To perform the recalibration, we used a linear model regressing apolipoprotein levels on batch as well as age, sex, center, alcohol, smoking, physical activity, and BMI, variables associated with apolipoprotein levels in our study population.

Outcome assessment

A standard 75g-OGTT was performed in the morning after an overnight fast at baseline and at 3-year follow-up in this study. Blood samples were taken at 0, 30, 60, 90, and 120 min after glucose ingestion. The plasma concentrations of glucose and insulin were measured for each time point. OGIS is an insulin sensitivity index calculated from OGTT measurements and is a validated estimate of the glucose clearance during a euglycemic-hyperinsulinemic clamp (21). The calculation is based on an equation derived from a regression model of the glucose-insulin relationship. In this paper, OGIS will be referred to as insulin sensitivity measured by OGTT (IS-OGTT).

On a separate day within one month of the baseline OGTT, a euglycemic-hyperinsulinemic clamp was performed. The clamp test was not repeated at 3 years. Insulin was administered as a primed-continuous infusion at a rate of 240 pmol/min/m² simultaneously with a variable 20% dextrose infusion adjusted every 5–10 min to maintain plasma glucose level within 0.8 mmol/L ($\pm 15\%$) of the target glucose level (4.5–5.5 mmol/L). Additional blood samples were obtained at 20-min intervals for insulin determination. The clamp procedure was standardized across centers. The M-I ratio is an insulin sensitivity index obtained from the clamp, which represents the amount of glucose metabolized per unit of plasma insulin. In this paper, the M-I ratio will be referred to as insulin sensitivity measured by clamp (IS-clamp).

Statistical analysis

Baseline characteristics of participants were described in men and women separately using medians for continuous variables and percentages for categorical variables.

The top and bottom 1% of the apolipoprotein exposures were trimmed to minimize the influence of outliers (n=20 removed). Observations that were missing any outcome

measurements (baseline or year 3 IS-OGIS or baseline IS-clamp) were deleted (n=97 removed). We used the extreme studentized deviate (ESD) procedure to identify and exclude outliers (n=3 removed) (22, 23) and excluded observations with residuals larger than 4 standard deviations (SDs) (n=3 removed). The final sample size was 864.

Linear regressions were performed to evaluate the relationships between apolipoprotein variables and outcomes. All outcome variables were log-transformed to improve normality. Sex-specific quintiles and quintile medians were created for each apolipoprotein variable, as baseline apolipoprotein levels were different between men and women. Tests for trend were based on quintile medians. Nonlinearity was assessed with likelihood-ratio tests, comparing a model treating quintile medians continuously to a model with quintile indicators. After confirming there were no deviations from linearity (all p for nonlinearity > 0.05), we also created sex-specific z-scores to analyze associations per SD. We back-transformed the coefficients to present results as the percent (%) difference in insulin sensitivity outcome per each SD higher or for each quintile compared with the lowest quintile. Potential confounders were selected based on our *a priori* knowledge as to the associations with the apolipoproteins as well as their effect on insulin sensitivity. Multivariable models were adjusted for age, sex, center, smoking (never smoked; previously smoked; currently smokes 1 to 14.9, ≥ 15 tobacco grams per day), alcohol consumption (women: 0, 0.1 to 4.9, 5.0 to 14.9, or ≥ 15.0 g per day; men: 0, 0.1 to 4.9, 5.0 to 29.9, or ≥ 30.0 g per day), physical activity (inactive; minimally active; highly active; or not reported) and BMI (continuous). For the main prospective analysis with IS-OGTT at year 3 as the outcome, log-transformed IS-OGTT at baseline was additionally adjusted as a covariate in the model. In this way, the model analyzes the 3-year change in IS-OGTT. Because apoA-I with and without apoC-III represent fractions of total apoA-I, the two apoA-I subspecies were entered

in the model simultaneously. Likewise, both apoB with and without apoC-III were included in the models for apoB subspecies. Likelihood ratio tests were performed based on linear trends to assess whether associations varied significantly for apoC-III-based lipoprotein subspecies. We ran additional regression models with the % of apoA-I with apoC-III simultaneously adjusting for the total concentration of apoA-I in the model. A model for apoC-III in HDL was also adjusted for total apoA-I. Multivariable models were additionally adjusted for triglycerides, which was considered a potential intermediate between apoC-III and insulin response. To investigate whether the associations we observed between apoA-I related measurements and insulin sensitivity are modified by risk factors for insulin resistance or diabetes, we tested for effect modification by sex, BMI (<20, 20-25, \geq 25 kg/m²), triglycerides (<150, \geq 150 mg/dl) and HDL-cholesterol (<40, 40-60, \geq 60 mg/dl) using likelihood ratio tests.

RESULTS

Study participant characteristics

The median age of study participants was 43 for men and 45 for women, and the median BMI was 26 kg/m² for men and 24 kg/m² for women. In general, participants had healthy lipid profiles, with high HDL cholesterol (median for men: 1.2 mmol/l, women: 1.5 mmol/l) and low LDL cholesterol (median for men: 3.0 mmol/l, women: 2.8 mmol/l) and triglycerides (median for men: 1.1 mmol/l, women: 0.8 mmol/l) (Table 1). During the 3-year follow-up, only 3 participants developed diabetes.

Associations between apolipoprotein concentrations and 3-year change in insulin sensitivity

ApoA-I in HDL with and without apoC-III measured at baseline demonstrated significantly opposite associations with 3-year change in IS-OGTT (p-heterogeneity=0.004) (Figure 2-1A, Table 2-2). Each 1 SD higher apoA-I without apoC-III (19 mg/dL for men and 22 mg/dL for women) at baseline was associated with a 1.3% improvement in IS-OGTT after 3 years (95% CI: 0.3, 2.2), while each 1 SD higher apoA-I with apoC-III (2.5 mg/dL for men and 2.7 mg/dL for women) was associated with a 1.2% decrease in IS-OGTT (95% CI: -2.1, -0.3). This was corroborated by the result from a model using the % of apoA-I with apoC-III as a main exposure, which was significantly associated with a decrease in IS-OGTT over 3 years (Figure 2-1B, Table 2-2). Total apoA-I was not significantly associated with a 3-year change in IS-OGTT.

ApoC-III concentration in HDL was strongly associated with a decrease in IS-OGTT over 3 years. For every SD higher apoC-III in HDL (2.5 mg/dL for men and 2.0 mg/dL for women), IS-OGTT decreased by 1.3% (95%CI: -2.1, -0.5) (Figure 2-1C, Table 2-2). The similar inverse associations were present but weaker for total apoC-III and the molar ratio of apoC-III to apoA-I.

None of the apoB measures (total apoB, apoB without apoC-III and apoB with apoC-III) were significantly associated with IS-OGTT assessed at year 3 (Supplementary Table 2-1). ApoC-III contained in apoB lipoproteins was weakly associated with a decrease in IS-OGTT; for every SD higher apoC-III in apoB lipoproteins, IS-OGTT decreased by 0.7% (95%CI: -1.5, 0.1).

Adjusting for triglycerides

All the significant associations between apolipoprotein levels and 3-year change in insulin sensitivity were attenuated and became null after triglycerides adjustment demonstrating

an apoC-III- triglycerides-insulin sensitivity pathway, as hypothesized (Supplementary Table 2-2).

Investigation of potential effect-modifiers

We found no evidence of effect modification by sex, BMI, triglycerides or HDL-cholesterol (P for interaction all >0.1).

Cross-sectional results for IS-clamp and IS-OGTT

We confirmed that total apoC-III, apoC-III in HDL and the apoC-III to apoA-I molar ratio were all significantly and inversely associated with both euglycemic-hyperinsulinemic clamp (IS-clamp) and IS-OGTT which were both available at the baseline exam. The order of strength of associations were similar for both endpoints (Figure 2-2A and B, Table 2-3). The apoC-III to apoA-I molar ratio had the strongest inverse association with both IS-clamp and IS-OGTT, followed by apoC-III in HDL. A weaker but still significantly inverse association was present between total apoC-III and both insulin sensitivity indexes.

DISCUSSION

The present study is the first to show that two HDL subspecies, HDL with and without apoC-III, have significantly opposite associations with changes in insulin sensitivity. We found that apoA-I without apoC-III was associated with improved insulin sensitivity, whereas apoA-I with apoC-III was associated with worsened insulin sensitivity over 3 years. These findings pertaining to apoC-III-containing HDL are strengthened by our findings on the apoC-III concentration in HDL, which was strongly associated with a reduction in insulin sensitivity.

Measuring the HDL subspecies or apoC-III in HDL is necessary to reveal these associations that involve apoC-III because plasma total apoA-I was unassociated and total apoC-III was only weakly associated with insulin sensitivity. This is because the apoB subspecies and apoC-III in apoB lipoproteins have a null and very weak association with insulin sensitivity and dilute the significant associations between apoC-III related HDL measurements and insulin sensitivity.

This and previous research indicate that the concept of HDL subspecies based on the presence or absence of apoC-III may have important implications for the risk of chronic diseases or metabolic conditions. The percentage of apoA-I in HDL that contains apoC-III in people with obesity was about 13-14%, which is double that of normal weight individuals (1). HDL with and without apoC-III demonstrated opposite associations with CHD risk in two prospective cohorts; HDL without apoC-III was associated with a decreased risk of CHD, whereas HDL with apoC-III was associated with an increased risk (2). In a cross-sectional analysis within the same study, higher concentrations of hemoglobin A1c (HbA1c) and prevalent diabetes were associated with higher levels of HDL with apoC-III and lower levels of HDL without apoC-III. Although measuring the concentration of the HDL subspecies is more complex than the apoC-III concentration in HDL, there is more information in the subspecies concerning age-related changes in insulin sensitivity because they show beneficial and harmful associations.

HDL and apoA-I are directly involved in glucose homeostasis (3-5), possibly by improving insulin secretion via reducing cholesterol accumulation in pancreatic β -cells (24, 25), increasing glucose uptake into skeletal muscle through the AMP-activated protein kinase signaling pathway (26, 27), improving insulin sensitivity via anti-inflammatory actions (28-30), and increasing insulin synthesis and secretion in pancreatic β -cells through incretin-like mechanisms (31). The present study demonstrated that the beneficial effects on insulin response

to glucose existed only for HDL without apoC-III, but not for HDL with apoC-III. Interestingly, total apoA-I levels were not associated with improvement in insulin sensitivity over 3 years, suggesting that the differential associations seen for HDL subspecies with and without apoC-III may be obscured by assessing only total apoA-I. There are potential mechanisms through which HDL with apoC-III might impair an otherwise favorable association of apoA-I in HDL with insulin sensitivity. HDL without apoC-III significantly inhibited human monocyte cell adhesion to cultured endothelial cells, *in vitro*, while HDL with apoC-III did not reduce adhesion (8), which suggests that the presence of apoC-III on HDL diminishes the anti-inflammatory effect of HDL without apoC-III. ApoC-III also promotes β -cell apoptosis by hyperactivation of β -cell CaV channels *in vitro* (11) and *in vivo* (10) and thus enhances insulin resistance.

In a previous analysis in RISC, total apoC-III levels measured by multiplex were found to be inversely associated with insulin sensitivity measured as IS-clamp at baseline (32). We confirmed this cross-sectional finding, using ELISA, with both insulin sensitivity indexes measured by clamp and OGTT. This is consistent with several other epidemiological studies, which demonstrated that apoC-III is associated with diabetes or metabolic syndrome cross-sectionally (14-16) and prospectively (17). Interestingly, we found that apoC-III in HDL was even more strongly and inversely associated with insulin sensitivity than total apoC-III levels in both cross-sectional and prospective analysis. This is consistent with a previous prospective cohort study on Turkish men and women, which has reported that apoC-III contained in HDL was associated with the risk of diabetes more strongly than total apoC-III (17). In another study, the concentration of apoC-III in HDL was significantly higher among obese people as compared to people with normal body weight (1). About 40% of apoC-III is in apoB lipoproteins which are very weakly associated with insulin sensitivity.

The results for apoB-lipoproteins were not significant. This might be because apoB-containing lipoproteins in this study contained both LDL and VLDL instead of only LDL. Mendivil et al 2011 showed that apoB in LDL with apoC-III was associated with increased risk of CHD, whereas the association between apoB in LDL without apoC-III and CHD risk was null (33). The same study showed no differential effects by VLDL subspecies, with both apoB in VLDL with and without apoC-III being associated with increased risk of CHD. In contrast, Lee et al 2003 showed that VLDL with apoC-III was associated with decreased risk for recurrent coronary events among diabetic patients, while VLDL without apoC-III was associated with increased risk (34). Similar to the present data from RISC, our unpublished data from the Danish Diet, Cancer and Health study cancer cohort study demonstrated similarly that total apoB (in both LDL and VLDL) with apoC-III was not associated with the incidence of diabetes. These results suggest a need to look at LDL and VLDL separately when examining their apoC-III defined subspecies with the risk of diabetes or CHD.

There are several strengths in our study. First, the RISC study is the largest study to date which used the gold standard technique, the euglycemic-hyperinsulinemic clamp, for measuring insulin sensitivity in healthy nondiabetic people. The euglycemic-hyperinsulinemic clamp was administered only at baseline, whereas OGTT was performed both at baseline and year 3. OGIS is a validated estimate of clamp-measured insulin sensitivity and we confirmed in this study that the two methods provided the same patterns of associations with apolipoprotein levels at baseline before we conducted the main prospective analysis using IS-OGTT at year 3. Second, the sandwich ELISA method has relatively low within-assay and between-assay variability compared to other methods such as immuno-affinity chromatography (Yamamoto et al 2015,

submitting). Finally, since all assays were performed by one analyst, there was no inter-rater variability.

Our study also has a number of limitations. Although we were able to perform prospective analysis, 3 years might not have been long enough for insulin sensitivity to substantially change, especially because the participants of this cohort were quite healthy at baseline. Another limitation is the variation across study sites due to the multi-center design of the RISC study. However, the exposure measurement was performed independently of the outcome assessment and blinded to the outcome status. Therefore, it is likely that the variation was non-differential and the effect estimates were pulled toward the null. Finally, the generalizability of the study was limited since the participants were all Europeans. Future studies using cohorts with different ethnic compositions are needed for improved generalizability.

In conclusion, in this prospective analysis of healthy men and women in the RISC cohort, two HDL subspecies had significantly opposite associations with 3-year change in insulin sensitivity, with HDL with apoC-III being associated with a decrease in insulin sensitivity and HDL without apoC-III associated with an increase in insulin sensitivity. In addition, the concentration of apoC-III contained in HDL had a strong and significant association with a decrease in insulin sensitivity over 3 years. Our results support the potential of HDL apoC-III as a promising target for diabetes prevention and treatment and have implications for a refined approach based on apoC-III content of future therapeutic targets for diabetes prevention.

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Table 2-1. Baseline characteristics of men and women in the RISC study included in this study

	Men		Women	
	Median	25th, 75th percentile	Median	25th, 75th percentile
Age, yrs	43	(36, 51)	45	(39, 51)
BMI, kg/m ²	26	(24, 28)	24	(22, 27)
Systolic BP, mmHg	123	(116, 130)	114	(105, 123)
Diastolic BP, mmHg	77	(71, 81)	73	(67, 78)
Physical activity (IPAQ), % (inactive/moderate/active)	20/42/39, %		20/44/37, %	
Alcohol, g/wk	76	(35, 143)	34	(11, 67)
Smoker, % (never/current/ex)	47/26/26, %		48/24/28, %	
Menopause, %	-		26%	
Fasting glucose, mmol/l	5.2	(4.9, 5.5)	5.0	(4.7, 5.3)
Fasting insulin, pmol/l	31	(22, 46)	30	(20, 41)
IS-clamp (baseline), μmol/min*kg_FFM*nM	116	(83, 153)	149	(111, 192)
IS-OGTT (baseline), ml/min*m ²	427	(392, 461)	447	(410, 488)
IS-OGTT (year 3), ml/min*m ²	414	(378, 451)	438	(393, 483)
delta_IS-OGTT, ml/min*m ²	-13	(-42, 18)	-8	(-46, 24)
Adiponectin, mg/l	6	(5, 8)	9	(7, 12)
Leptin, ng/ml	5	(2, 8)	15	(10, 25)
Cholesterol, mmol/l	4.9	(4.4, 5.5)	4.8	(4.2, 5.3)
HDL-C, mmol/l	1.2	(1.1, 1.4)	1.5	(1.3, 1.8)
LDL-C, mmol/l	3.0	(2.6, 3.6)	2.8	(2.3, 3.3)
TG, mmol/l	1.1	(0.8, 1.5)	0.8	(0.6, 1.1)
FFA, mmol/l	0.43	(0.35, 0.57)	0.56	(0.45, 0.72)
ApoA-I without apoC-III, mg/dl	90	(78, 103)	103	(89, 118)
ApoA-I with apoC-III, mg/dl	7.4	(6.0, 8.9)	8.4	(7.0, 10.4)
% of apoA-I with apoC-III, %	7.5	(6.4, 8.9)	7.6	(6.6, 8.8)
ApoB without apoC-III, mg/dl	71	(61, 87)	67	(55, 80)
ApoB with apoC-III, mg/dl	4.3	(3.3, 5.5)	4.0	(3.1, 5.1)
% of apoB with apoC-III, %	5.6	(4.5, 6.9)	5.8	(4.6, 7.0)
ApoC-III, mg/dl	9.9	(7.6, 12.8)	9.9	(7.6, 12.3)

ApoC-III in HDL, mg/dl	5.8	(4.6, 7.6)	5.7	(4.6, 7.0)
ApoC-III in apoB, mg/dl	4.1	(2.8, 6.0)	4.2	(2.8, 5.6)
ApoC-III : ApoA-I molar ratio	2.6	(2.2, 3.1)	2.2	(1.9, 2.6)
ApoC-III : ApoB molar ratio	63	(43, 80)	62	(47, 84)

BMI=body mass index, IPAQ=international physical activity questionnaire, BP=blood pressure, FFM=fat free mass, IS-clamp=insulin sensitivity measured by euglycemic-hyperinsulinemic clamp, IS-OGTT=insulin sensitivity measured by oral glucose tolerance test, HDL-C=HDL-Cholesterol, LDL-C=LDL-Cholesterol, TG=triglycerides, FFA=free fatty acid, ApoA-I without apoC-III=the concentration of apoA-I in HDL that does not contain apoC-III, ApoA-I with apoC-III=the concentration of apoA-I in HDL that contains apoC-III, % of apoA-I with apoC-III=% of apoA-I in HDL that contains apoC-III, ApoB without apoC-III=the concentration of apoB in LDL and VLDL that does not contain apoC-III, ApoB with apoC-III=the concentration of apoB in LDL and VLDL that contains apoC-III, % of apoB with apoC-III=% of apoB in LDL and VLDL that contains apoC-III, ApoC-III in HDL=the concentration of apoC-III contained in HDL fraction, ApoC-III in apoB=the concentration of apoC-III contained in LDL and VLDL, ApoC-III : ApoA-I molar ratio=the molar ratio of apoC-III to apoA-I in apoC-III-containing HDL, ApoC-III : ApoB molar ratio= the molar ratio of apoC-III to apoB in apoC-III-containing LDL and VLDL

Table 2-2. Association between apolipoprotein concentrations in HDL and 3-year change in insulin sensitivity

Outcome	Exposure	Quintiles										P-trend	per SD	
		Q1	Q2		Q3		Q4		Q5		P		% difference	P
		ref	% difference	P	% difference	P	% difference	P	% difference	P				
IS-OGTT (year 3)	Total apoA-I	0%	-0.5%	0.68	1.7%	0.14	2.3%	0.047	0.5%	0.65	0.25	0.4%	0.26	
	ApoA-I without apoC-III	0%	1.3%	0.28	2.3%	0.051	4.6%	0.0004	2.8%	0.04	0.01	1.3%	0.01	
	ApoA-I with apoC-III	0%	-0.4%	0.73	-1.2%	0.30	-1.0%	0.42	-3.0%	0.03	0.02	-1.2%	0.01	
	% apoA-I with apoC-III	0%	-1.3%	0.24	-0.6%	0.57	-1.9%	0.09	-2.6%	0.02	0.02	-1.0%	0.005	
	Total apoC-III	0%	1.1%	0.33	-0.5%	0.69	0.0%	0.97	-1.8%	0.11	0.049	-0.6%	0.11	
	ApoC-III in HDL	0%	-0.3%	0.77	-0.6%	0.63	-1.6%	0.18	-3.7%	0.004	0.002	-1.3%	0.002	
	ApoC-III to apoA-I ratio	0%	-0.8%	0.49	-1.2%	0.31	-1.6%	0.18	-2.1%	0.10	0.08	-0.8%	0.04	

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI. Adjusted for log-transformed IS-OGTT at baseline. Analysis of apoA-I without and with apoC-III simultaneously adjusted. Analysis of % apoA-I with apoC-III and apoC-III in HDL also adjusted for total apoA-I.

Table 2-3. Cross-sectional association between apolipoprotein concentrations in HDL and insulin sensitivity at baseline

Outcome	Exposure	Quintiles									P-trend	per SD		
		Q1	Q2		Q3		Q4		Q5			P	% difference	P
		ref	% difference	P	% difference	P	% difference	P	% difference	P				
IS-clamp	Total apoC-III	0%	1.1%	0.80	-3.3%	0.42	-3.5%	0.40	-6.3%	0.12	0.04	-3.0%	0.02	
	ApoC-III in HDL	0%	-5.2%	0.19	-6.9%	0.09	-9.1%	0.03	-14.8%	0.0004	0.0003	-5.5%	0.0001	
	ApoC-III to apoA-I ratio	0%	-10.0%	0.01	-8.6%	0.03	-18.4%	<0.0001	-22.3%	<0.0001	<0.0001	-7.9%	<0.0001	
IS-OGTT	Total apoC-III	0%	1.0%	0.42	-0.6%	0.63	-0.4%	0.75	-1.6%	0.19	0.07	-0.8%	0.04	
	ApoC-III in HDL	0%	-0.3%	0.81	-0.6%	0.65	-1.5%	0.23	-3.2%	0.02	0.008	-1.5%	0.0008	
	ApoC-III to apoA-I ratio	0%	-2.6%	0.04	-3.3%	0.007	-4.8%	0.0001	-6.2%	<0.0001	<0.0001	-2.1%	<0.0001	

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI.

Supplementary Table 2-1. Association between apolipoprotein concentrations in LDL and VLDL and 3-year change in insulin sensitivity

Outcome	Exposure	Quintiles										P-trend	per SD	
		Q1	Q2		Q3		Q4		Q5		P		% difference	P
		ref	% difference	P	% difference	P	% difference	P	% difference	P				
IS-OGTT (year 3)	Total apoB	0%	-2.4%	0.04	-0.5%	0.67	-1.1%	0.36	-2.1%	0.09	0.25	-0.5%	0.18	
	ApoB without apoC-III	0%	-1.7%	0.16	0.1%	0.96	-0.9%	0.50	-1.4%	0.32	0.52	-0.3%	0.53	
	ApoB with apoC-III	0%	1.1%	0.36	1.2%	0.32	-0.9%	0.46	-0.2%	0.89	0.45	-0.4%	0.33	
	% apoB with apoC-III	0%	0.0%	0.97	2.2%	0.06	-0.5%	0.69	-0.1%	0.94	0.78	-0.5%	0.22	
	ApoC-III in apoB	0%	-1.0%	0.41	-1.2%	0.32	-2.2%	0.08	-2.2%	0.08	0.048	-0.7%	0.09	

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI. Adjusted for log-transformed IS-OGTT at baseline. Analysis of apoB without and with apoC-III simultaneously adjusted. Analysis of % apoB with apoC-III and apoC-III in apoB also adjusted for total apoB.

Supplementary Table 2-2. Association between apolipoprotein concentrations in HDL and 3-year change in insulin sensitivity, additional TG adjustment

Outcome	Exposure	Quintiles									P-trend	per SD	
		Q1	Q2		Q3		Q4		Q5			% difference	p
		ref	% difference	p	% difference	p	% difference	p	% difference	p			
IS-OGTT (year 3)	Total apoA-I	0%	-0.5%	0.64	1.5%	0.19	2.1%	0.07	0.6%	0.63	0.25	0.4%	0.24
	ApoA-I without apoC-III	0%	1.0%	0.40	1.8%	0.13	3.7%	0.004	1.9%	0.17	0.08	0.9%	0.07
	ApoA-I with apoC-III	0%	0.1%	0.96	0.6%	0.65	0.2%	0.87	1.6%	0.29	0.29	-0.6%	0.21
	% apoA-I with apoC-III	0%	-1.1%	0.35	-0.3%	0.78	-1.5%	0.18	-1.2%	0.33	0.31	-0.6%	0.14
	Total apoC-III	0%	1.5%	0.21	0.3%	0.83	0.6%	0.62	0.2%	0.87	0.99	0.4%	0.43
	ApoC-III in HDL	0%	-0.1%	0.96	0.0%	0.97	-1.0%	0.44	-1.9%	0.22	0.25	-0.6%	0.27
	ApoC-III to apoA-I ratio	0%	-0.5%	0.70	-0.9%	0.43	-0.8%	0.54	-0.5%	0.72	0.80	-0.2%	0.65

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity, BMI and TG. Adjusted for log-transformed IS-OGTT at baseline. Analysis of apoA-I without and with apoC-III simultaneously adjusted. Analysis of % apoA-I with apoC-III and apoC-III in HDL also adjusted for total apoA-I.

Figure 2-1. Association between apolipoprotein concentrations in HDL and 3-year change in insulin sensitivity

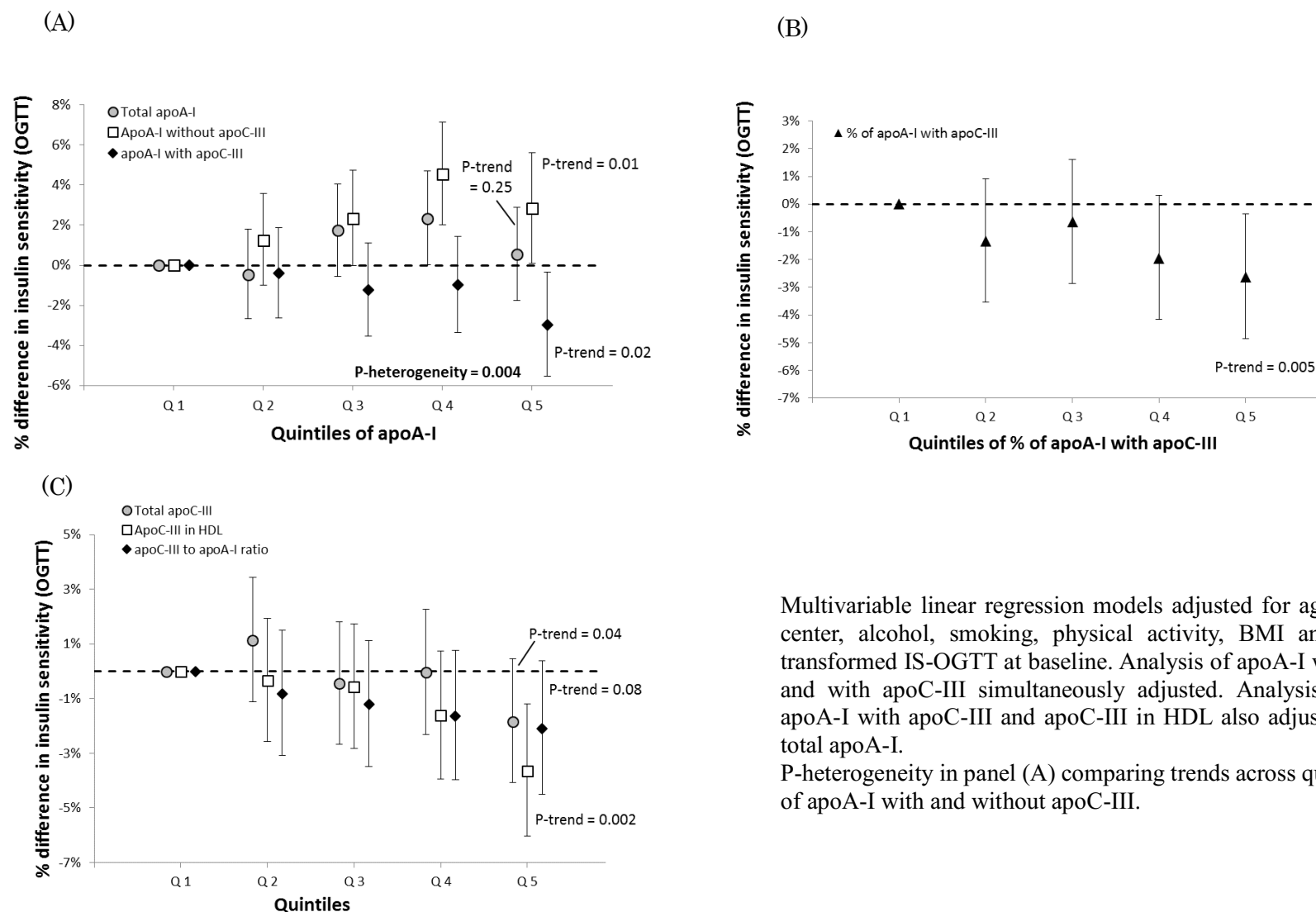
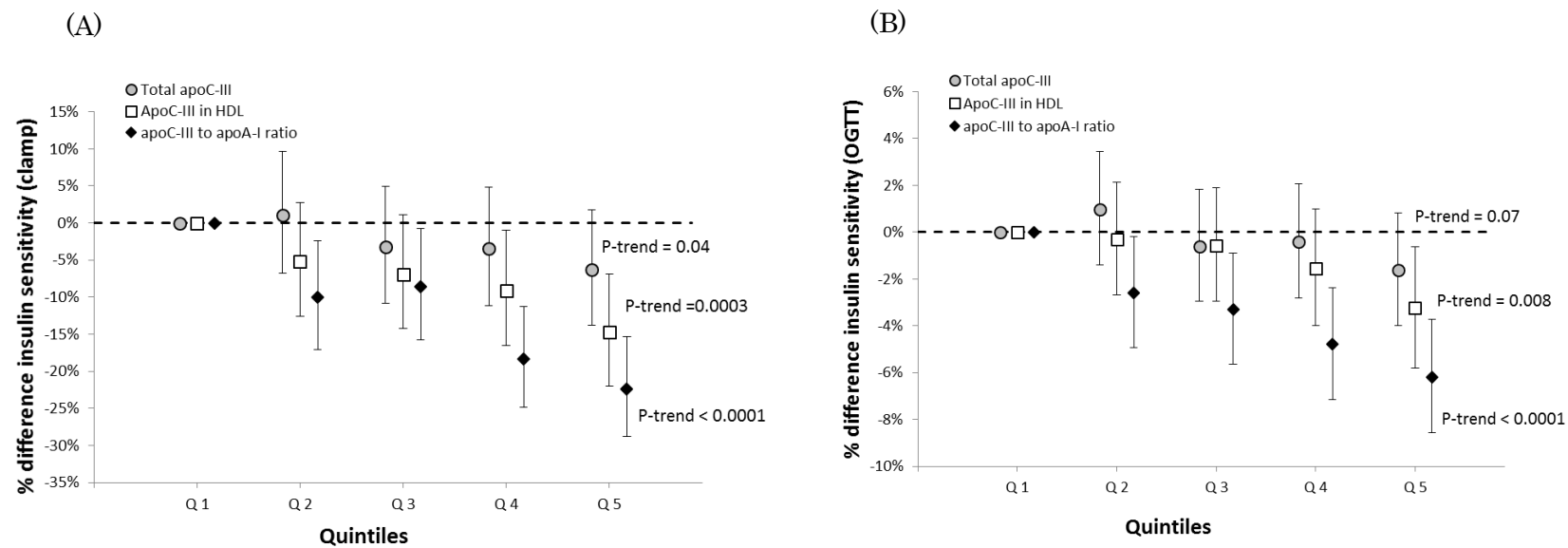


Figure 2-2. Cross-sectional association between apolipoprotein concentrations in HDL and insulin sensitivity at baseline



Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI.

Chapter 3

High Density Lipoprotein with apolipoprotein C-III is associated with early carotid atherosclerosis

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ABSTRACT

Background

About 6-7% of High Density Lipoprotein (HDL) has apolipoprotein C-III (apoC-III), a protein known for its proinflammatory properties. When apoC-III is present on HDL, HDL loses its protective inverse association with risk of coronary heart disease (CHD), whereas HDL without apoC-III is strongly inversely associated with CHD. We investigated how the presence of apoC-III affects the association between HDL and early stages of atherosclerosis measured as carotid intima-media thickness (cIMT).

Methods

We examined the cross-sectional associations between the apoA-I concentrations of HDL subspecies with and without apoC-III and cIMT among 847 participants from the European multi-center “*Relationship between Insulin Sensitivity and Cardiovascular disease*” (RISC) study. The concentrations of these apoC-III-based HDL subspecies were measured by sandwich ELISA. cIMT at common carotid artery (cIMT at CCA) and the maximum cIMT (cIMT max) were measured by high resolution B-mode carotid ultrasonography.

Findings

HDL with and without apoC-III demonstrated significantly opposite associations with both cIMT indexes (p-heterogeneity of associations comparing the two subspecies was 0.002 for cIMT at CCA and 0.006 for cIMT max). Compared to the lowest quintile, the highest quintile of apoA-I without apoC-III was associated with 3.7% lower cIMT at CCA (p-trend=0.01) or 7.3% lower cIMT max (p-trend=0.003), while the highest quintile of apoA-I with apoC-III was associated with 4.4% higher cIMT at CCA (p-trend=0.001) or 7.9% higher cIMT max

(p-trend=0.002). Total apoA-I was not significantly associated with either cIMT indexes (p-trend>0.5), whereas higher levels of total apoC-III and apoC-III contained in HDL were significantly associated with higher cIMT (p-trend<0.01)

Interpretation

Both the concentration of HDL that has apoC-III and the concentration of apoC-III itself in HDL were associated with greater atherosclerosis in the carotid artery. ApoC-III-based HDL subspecies may be more informative than total HDL in distinguishing HDL functions and support the potential of HDL apoC-III as a promising target for atherosclerosis prevention and treatment.

INTRODUCTION

A low level of HDL cholesterol or apolipoprotein A-I is a major risk factor for atherosclerotic cardiovascular diseases (1-4). However, the atheroprotective effect of HDL cholesterol is challenged by genetic studies and pharmaceutical trials, which do not support the causal relationship between increased HDL cholesterol levels and CVD. These findings suggest that HDL cholesterol might not fully capture the anti-atherogenic functions of HDL.

The protein component of HDL rather than the cholesterol content of HDL might be more informative in explaining HDL functions because properties of these proteins can modify the functions of HDL. We recently found that HDL exists in subspecies based on the content of a small protein called apolipoprotein C-III (apoC-III). ApoC-III resides on the surface of lipoprotein particles and is shown to provoke inflammatory and atherogenic responses in monocytes and endothelial cells (5). Reilly and colleagues reported that apoC-III was associated with coronary artery calcification (CAC), a measure of asymptomatic atherosclerosis, in a cross-sectional study of persons with type 2 diabetes (11). About 6-7% of the total plasma apoA-I, the main HDL apolipoprotein, has apoC-III in healthy people with normal body weight (6), while the remainder of apoA-I is free of apoC-III. The percentage of apoA-I HDL that contains apoC-III in people with obesity is about 13-14%, which is double that of normal weight individuals (6). Furthermore, HDL with and without apoC-III demonstrated opposite associations with CHD risk in two prospective cohorts; HDL without apoC-III was associated with decreased risk of CHD, whereas HDL with apoC-III was associated with increased risk of CHD (7).

Carotid intima-media thickness (cIMT) is a marker for early stage atherosclerosis (8) that is associated with cardiovascular risk factors (9) and cardiovascular outcomes (10). In this paper, we investigated how apoC-III-based HDL subspecies are associated with subclinical carotid atherosclerosis assessed as cIMT in a European multi-center study. We

hypothesized that HDL with apoC-III would be positively associated with cIMT, while HDL without apoC-III would be inversely associated with cIMT. We also studied apoB (includes LDL and VLDL lipoproteins) subspecies based on the presence of apoC-III. Based on a previous study (12), we hypothesized that apoB lipoproteins without apoC-III would be positively associated with cIMT but that associations for apoB with apoC-III would be even stronger.

MATERIALS AND METHODS

Study design and participants

RISC is a European multi-center prospective cohort study with a standardized protocol and a centralized evaluation of measures (13). Healthy men and women ages 30-60 (n=1,513) were recruited between June 2002 and July 2004, from the local population at 19 centers in 14 European countries (Austria, Denmark, Finland, France, Germany, Greece, The Netherlands, Ireland, Italy, Sweden, Spain, Switzerland, United Kingdom and Serbia and Montenegro). Follow-up examinations were performed after 3 years. Exclusion criteria were treatment for any chronic disease, pregnancy, any cardiovascular event, weight change of ≥ 5 kg in the last 3 months, cancer in the last 5 years, and renal or liver failure, recent major surgery, seizure disorder or epilepsy, arterial blood pressure $\geq 140/90$ mmHg, fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dl), 2-hour plasma glucose (on a standard 75-g OGTT) ≥ 11.0 mmol/L (200 mg/dl), total serum cholesterol ≥ 7.8 mmol/L (300 mg/dl), serum triglycerides ≥ 4.6 mmol/L (400 mg/dl), electrocardiogram abnormalities or acute myocardial ischemia injury or pericarditis. Out of 1,017 eligible subjects who had several biomarkers measured by the RISC investigators, baseline plasma samples of 987 subjects, which had enough plasma left for our experiment, were sent to our laboratory. Each recruiting center obtained approval from local ethics committee.

Exposure assessment

Information on basic characteristics of the participants including demographics (age, sex) and lifestyle (smoking, alcohol consumption, physical activity based on the International Physical Activity Questionnaire (IPAQ) score) were collected by interviews and questionnaires. Anthropometric measures and lipid levels (LDL-, HDL-, total-cholesterol and triglycerides) were also measured (13).

We used a novel sandwich ELISA that we recently developed to measure the concentrations of total apoC-III, apoA-I in HDL without apoC-III, apoA-I in HDL with apoC-III; and the concentrations of apoB in lipoproteins with and without apoC-III (Yamamoto et al 2015, submitted). Briefly, a 96-well microplate coated with anti-human apoC-III antibody captured plasma lipoproteins that contained apoC-III. The unbound fraction was transferred to another plate coated with anti-human apoA-I antibody to measure the concentration of apoA-I that was not associated with apoC-III. The lipoproteins bound to the anti-apoC-III plate were dissociated with Tween-20, and then transferred to a plate coated with anti-human apoA-I antibody to measure the concentration of apoA-I that contained apoC-III. Total apoA-I was calculated as the sum of the concentrations of apoA-I with and without apoC-III. The concentrations of apoB with and without apoC-III were measured by applying the same principle for the apoA-I subspecies.

For the measurement of apoC-III in HDL, whole plasma was precipitated with reagent to remove apoB-containing lipoproteins, and the apoC-III concentration in the remaining fraction was measured by standard sandwich ELISA. ApoC-III in HDL is different from apoA-I with apoC-III; the former represents the concentration of apoC-III itself contained in HDL whereas the latter represents the concentration of apoA-I in HDL with *any* amount of apoC-III. The apoC-III to apoA-I molar ratio, which may be interpreted as the density of

apoC-III on apoC-III-containing HDL, was calculated as the number of apoC-III molecules per apoA-I molecule on HDL particles containing apoC-III.

Apolipoprotein measurements were corrected for batch variability using methods developed by Rosner et al (14). Age, sex, center, alcohol, smoking, physical activity and BMI were included in the regression for the Rosner correction method and were chosen based on the association with the exposure and the outcome.

Outcome assessment

Carotid images were obtained in each center by B-mode high-resolution ultrasonography, with the participant supine with neck slightly extended and head rotated contralaterally to the side. Longitudinal B-mode image was obtained of the distal 10 mm of right and left common carotid arteries, carotid bifurcation, and internal carotid artery from anterior, lateral and posterior angles. cIMT reading was performed in a centralized center (Pisa) by a single reader blinded to clinical data. cIMT was measured by bow compasses in digitized zoomed diastolic frames of each carotid segment at 5 different points and the average was calculated for each segment. For statistical analysis, cIMT at CCA was calculated as the mean of each CCA segment. cIMT max is the maximum cIMT in any carotid segment.

As cIMT changes vary little over a short time, we treated the cIMT measures from baseline and year 3 as repeats and calculated their average to obtain most stable estimates of cIMT in a cross-sectional analysis. Additionally, we created a composite index by averaging cIMT at CCA and cIMT max.

Statistical analysis

Baseline characteristics of participants were described in men and women separately using medians for continuous variables and percentages for categorical variables.

The top and bottom 2% of the apolipoprotein exposures were trimmed to minimize the influence of outliers (n=40 removed). Observations that were missing outcome measurements, cIMT at CCA or cIMT max at baseline were deleted (n=83 removed). We used the extreme studentized deviate (ESD) procedure to identify and exclude outliers (15, 16) (n=15 removed) and excluded observations with residuals larger than 4 standard deviation (SD) (n=2 removed). The final sample size was 847.

Linear regressions were performed to evaluate the relationships between apolipoprotein variables and outcomes. All outcome variables were log-transformed to improve normality. Sex-specific quintiles and quintile medians were created for each apolipoprotein variable, as the baseline apolipoprotein levels were different between men and women. Tests for trend were based on quintile medians. Nonlinearity was assessed with likelihood-ratio tests, comparing a model treating quintile medians continuously to a model with quintile indicators. In case there were no deviations from linearity (p for nonlinearity > 0.05), we also created sex-specific z-scores to analyze associations per SD. We back-transformed the coefficients to present results as the percent (%) difference in cIMT outcome per each SD higher or for each quintile compared with the lowest quintile. Potential confounders were selected based on our *a priori* knowledge as to the associations with the apolipoproteins as well as their effect on cIMT. Multivariable models were adjusted for age, sex, center, smoking (never; past; current smoker 1 to 14.9 or ≥ 15 tobacco grams per day), alcohol consumption (women: 0, 0.1 to 4.9, 5.0 to 14.9, or ≥ 15.0 g per day; men: 0, 0.1 to 4.9, 5.0 to 29.9, or ≥ 30.0 g per day), physical activity (inactive; minimally active; highly active; or not reported) and BMI (continuous). Because apoA-I with and without apoC-III represent fractions of total apoA-I, the two apoA-I subspecies were entered in the model

simultaneously. Likewise, both apoB with and without apoC-III were included in the models for apoB lipoproteins. Likelihood ratio tests were performed based on linear trends to assess whether associations varied significantly for apoC-III-based subspecies. A model for apoC-III in HDL was adjusted for total apoA-I. Finally, multivariable models were adjusted for triglycerides in the additional analysis.

RESULTS

Study participant characteristics

The median age of study participants was 43 for men and 45 for women, and the median BMI was 26 kg/m² for men and 24 kg/m² for women. In general, participants had healthy lipid profiles with high HDL cholesterol (median for men: 1.2 mmol/l, women: 1.5 mmol/l) and low LDL cholesterol (median for men: 3.0 mmol/l, women: 2.8 mmol/l) and triglycerides (median for men: 1.1 mmol/l, women: 0.9 mmol/l) (Table 3-1). cIMT values did not substantially change over the 3-year follow-up period. Median cIMT at CCA changed from 0.61 mm to 0.62 mm in men and from 0.59 mm to 0.60 mm in women, and median cIMT max remained at 0.93 mm in men and changed from 0.88 mm to 0.90 mm in women.

Associations between apolipoprotein A-I concentrations and cIMT

ApoA-I in HDL with and without apoC-III demonstrated significantly opposite associations with both cIMT indexes in multivariable-adjusted models (p-heterogeneity of associations comparing the two subtypes was 0.002 for cIMT at CCA and 0.006 for cIMT max) (Figure 3-1). Compared to the lowest quintile, the highest quintile of apoA-I without apoC-III was associated with 3.7% lower cIMT at CCA (p-trend=0.01) or 7.3% lower cIMT max (p-trend=0.003), while the highest quintile of apoA-I with apoC-III was associated with 4.4% higher cIMT at CCA (p-trend=0.001) or 7.9% higher cIMT max (p-trend=0.002) (Table

3-2). Total apoA-I was not significantly associated with either cIMT indexes (p-trend>0.5). These observations were consistent with the per SD analysis. Each SD higher apoA-I without apoC-III (= 18 mg/dL for men and 20 mg/dL for women) was associated with 1.0% lower cIMT at CCA (95% CI: -1.9, -0.1%) or 2.1% lower cIMT max (95% CI: -3.8, -0.4%), while each SD higher apoA-I with apoC-III (= 2.5 mg/dL for men and 2.6 mg/dL for women) was associated with 1.5% higher cIMT at CCA (95% CI: 0.6, 2.4%) or 2.2% higher cIMT max (95% CI: 0.5, 4.0%). These associations were confirmed when the composite cIMT was used as outcome (Figure 3-1C, Table 3-2). After additional adjustment of triglycerides in the multivariate model, the associations were attenuated but apoA-I with and without apoC-III still demonstrated significantly opposite associations with all cIMT indexes (Supplementary Table 3-1).

Associations between apolipoprotein C-III concentrations and cIMT

ApoC-III concentration in HDL was associated with higher cIMT. The highest quintile of apoC-III in HDL was associated with 4.6% higher cIMT at CCA (p-trend=0.0003) or 5.9% higher cIMT max (p-trend=0.02) as compared to the lowest quintile (Figure 3-2, Table 3-3). These significant associations persisted even after adjusting for triglycerides. Total apoC-III was also associated with cIMT at CCA (p-trend=0.02) and composite cIMT (p-trend=0.045). However, after additionally adjusting for triglycerides, these associations became null (Supplementary Table 3-2). The apoC-III to apoA-I molar ratio was not associated with any of cIMT indexes. All the above findings were consistent between the quintile analysis and per SD analysis.

ApoB subspecies concentrations

Total apoB was associated with higher cIMT. The highest quintile had 3.3% higher cIMT at CCA (p-trend=0.004) or 4.3% higher cIMT max (p-trend=0.047). Although the associations were not as strong and consistent as those of total apoB, apoB without apoC-III was also associated with cIMT at CCA and the composite cIMT, especially in the per SD analysis. No such associations were present for apoB with apoC-III (Table 3-4).

DISCUSSION

The present study showed that two HDL subspecies, HDL with and without apoC-III, have significantly opposite associations with cIMT. We found that apoA-I without apoC-III was associated with lower cIMT, whereas apoA-I with apoC-III was associated with higher cIMT. These findings pertaining to apoC-III-containing HDL are strengthened by our findings on apoC-III in HDL, which was positively associated with cIMT, because it reiterates that the apoC-III component of HDL is crucial in determining the association of HDL with cIMT. This and previous research indicate that the concept of HDL subspecies based on the presence or absence of apoC-III may have implications for metabolic conditions and CHD risk (6, 7).

We did not observe an association between total HDL levels, reflected by measures of plasma total apoA-I, and cIMT. This is in agreement with previous studies which reported that low HDL cholesterol levels due to mutations in ABCA1, APOAI and LCAT were not associated with increased cIMT and high HDL cholesterol levels due to CETP deficiency were not associated with cIMT (17). Results of observational studies are also conflicting with regard to the association between HDL cholesterol and cIMT (18). The Carotid Atherosclerosis Progression Study among 3,383 participants reported that HDL cholesterol levels were not associated with any of cIMT measured at CCA, internal carotid artery and carotid bifurcation (19). In Multi-Ethnic Study of Atherosclerosis (MESA) study with 4,792

participants, low HDL cholesterol levels were significantly associated with cIMT max but not with cIMT measured at internal carotid artery (20).

There are a few potential mechanisms through which apoC-III may render HDL proatherogenic. Studies in cells and animal models have shown that apoC-III increases cytokine expression in cultured monocytes and endothelial cells (5, 21, 22). HDL without apoC-III decreased the proinflammatory adhesion of human monocytes to endothelial cells, whereas HDL with apoC-III did not (21). ApoC-III inhibits clearance of plasma VLDL and LDL by the liver (23-26). ApoC-III may similarly affect the circulation of HDL and its delivery to the liver. However, this needs to be confirmed by kinetics studies to compare the catabolism of HDL with and without apoC-III. We also speculate that apoC-III may interfere with the cholesterol efflux capacity of HDL. In two distinct cohorts of 996 patients, cholesterol efflux capacity was inversely associated with cIMT independently of HDL cholesterol and apoA-I levels (27). Interestingly, HDL cholesterol was significantly associated with cholesterol efflux capacity but was not associated with cIMT. It would be interesting to perform cholesterol efflux assay using HDL separated based on the presence of apoC-III and investigate if HDL subspecies have differential effects on cholesterol efflux.

The results for apoB-lipoprotein subspecies were not significant. This might be because apoB-containing lipoproteins in this study contained both LDL and VLDL instead of only LDL. Mendivil et al showed that apoB in LDL with apoC-III was associated with increased risk of CHD, whereas the association between apoB in LDL without apoC-III and CHD risk was null (12). The same study showed no differential effects by VLDL subspecies, with both apoB in VLDL with and without apoC-III being associated with increased risk of CHD. In contrast, Lee et al 2003 showed that the apoB concentration of VLDL with apoC-III was associated with decreased risk for recurrent coronary events among diabetic patients, while VLDL without apoC-III was associated with increased risk (28). Taken together, these

results suggest a need to look at LDL and VLDL separately when examining their apoC-III defined subspecies with the risk of CHD.

There are several strengths in our study. First, as we used replicated measurements for cIMT outcome values, measurement errors were reduced. Since the measurement errors were most likely non-differential, the effect estimates became more precise. Second, cIMT was measured in two different ways, cIMT at CCA and cIMT max. We also created a composite index of the two. We were able to confirm consistent results overall across the three cIMT indexes. Third, the modified sandwich ELISA method has relatively low intra-assay and inter-assay variability compared to other methods such as immuno-affinity chromatography (Yamamoto et al 2015, submitting). Finally, since all assays for apolipoprotein exposures and cIMT reading were performed by one analyst each, there was no inter-rater variability.

One limitation of this study is the cross-sectional nature of analysis and therefore the temporality of the associations could not be established. Another limitation is the variation across study sites due to the multi-center design of the RISC study. However, the exposure measurement was performed independently of the outcome assessment and blinded to the outcome status. Therefore, it is likely that the variation was non-differential and the effect estimates were pulled toward the null. Finally, the generalizability of the study was limited since the participants were all Europeans. Future studies using cohorts with different ethnic compositions are needed for improved generalizability.

In conclusion, in the analysis of healthy men and women in the RISC cohort, two HDL subspecies had significantly opposite associations with cIMT, with HDL with apoC-III being associated with higher cIMT and HDL without apoC-III associated with lower cIMT. In addition, the concentration of apoC-III contained in HDL was associated with higher cIMT. Our results support the potential of HDL apoC-III as a promising target for

atherosclerosis prevention and treatment and have implications for a refined approach based on apoC-III content of future therapeutic targets for cardiovascular prevention.

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The RISC Study

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Table 3-1. Baseline characteristics of men and women in the RISC study included in this study

	Men (N=369)		Women (N=478)	
	Median	25th, 75th percentile	Median	25th, 75th percentile
Age, yrs	43	(36, 51)	45	(39, 51)
BMI, kg/m ²	26	(24, 28)	24	(22, 27)
Systolic BP, mmHg	122	(115, 130)	115	(106, 123)
Diastolic BP, mmHg	77	(71, 81)	73	(67, 79)
IPAQ, % (inactive/moderate/active)	19/42/39, %		19/43/37, %	
Alcohol, g/wk	77	(38, 143)	30	(11, 65)
Smoker, % (never/current/ex)	47/27/26, %		47/25/28, %	
Menopause, %	-		26%	
Average of cIMT at CCA, mm	0.61	(0.56, 0.67)	0.59	(0.54, 0.65)
cIMT at CCA (baseline), mm	0.61	(0.56, 0.66)	0.59	(0.53, 0.64)
cIMT at CCA (year 3), mm	0.62	(0.57, 0.69)	0.60	(0.56, 0.66)
Average of cIMT max, mm	0.94	(0.84, 1.11)	0.89	(0.80, 1.05)
cIMT max (baseline), mm	0.93	(0.83, 1.11)	0.88	(0.78, 1.07)
cIMT max (year 3), mm	0.93	(0.82, 1.13)	0.90	(0.80, 1.05)
Adiponectin, mg/l	6	(5, 8)	9	(7, 12)
Leptin, ng/ml	5	(2, 8)	15	(10, 24)
Cholesterol, mmol/l	4.9	(4.3, 5.5)	4.8	(4.3, 5.4)
HDL-C, mmol/l	1.2	(1.1, 1.4)	1.5	(1.4, 1.8)
LDL-C, mmol/l	3.0	(2.6, 3.6)	2.8	(2.3, 3.3)
TG, mmol/l	1.1	(0.8, 1.5)	0.9	(0.6, 1.1)
FFA, mmol/l	0.43	(0.35, 0.57)	0.56	(0.45, 0.71)
ApoA-I without apoC-III, mg/dl	90	(79, 102)	103	(90, 117)
ApoA-I with apoC-III, mg/dl	7.4	(6.1, 8.9)	8.4	(7.0, 10.1)
% of apoA-I with apoC-III, %	7.6	(6.5, 8.9)	7.6	(6.6, 8.8)
ApoB without apoC-III, mg/dl	71	(61, 86)	67	(55, 80)
ApoB with apoC-III, mg/dl	4.3	(3.3, 5.5)	4.0	(3.1, 5.0)
% of apoB with apoC-III, %	5.6	(4.6, 6.9)	5.8	(4.7, 7.0)
ApoC-III, mg/dl	9.9	(7.7, 12.8)	9.8	(7.6, 12.3)
ApoC-III in HDL, mg/dl	5.8	(4.7, 7.7)	5.6	(4.6, 6.9)
ApoC-III in apoB, mg/dl	4.2	(2.8, 6.0)	4.2	(2.8, 5.7)
ApoC-III : ApoA-I molar ratio	2.6	(2.2, 3.1)	2.2	(1.9, 2.6)
ApoC-III : ApoB molar ratio	61	(43, 80)	62	(47, 84)

BMI=body mass index, BP=blood pressure, IPAQ=international physical activity questionnaire, cIMT at CCA=carotid intima-media thickness at common carotid artery, cIMT max=the maximum carotid intima-media thickness, FFM=fat free mass, HDL-C= HDL Cholesterol, LDL-C=LDL Cholesterol, TG=triglyceride, FFA=free fatty acid, ApoA-I without apoC-III=the concentration of apoA-I in HDL that does not contain apoC-III, ApoA-I with apoC-III=the concentration of apoA-I in HDL that contains apoC-III, % of apoA-I with apoC-III=% of apoA-I in HDL that contains apoC-III, ApoB without apoC-III=the concentration of apoB in LDL and VLDL that does not contain apoC-III, ApoB with apoC-

III=the concentration of apoB in LDL and VLDL that contains apoC-III, % of apoB with apoC-III=% of apoB in LDL and VLDL that contains apoC-III, ApoC-III in HDL=the concentration of apoC-III contained in HDL fraction, ApoC-III in apoB=the concentration of apoC-III contained in LDL and VLDL, ApoC-III : ApoA-I molar ratio= the molar ratio of apoC-III to apoA-I in apoC-III-containing HDL, ApoC-III : ApoB molar ratio= the molar ratio of apoC-III to apoB in apoC-III-containing LDL and VLDL

Table 3-2. Association between apoA-I concentrations and cIMT

Outcome	Exposure	Quintiles										P-trend	per-SD	
		Q1	Q2		Q3		Q4		Q5		P		% difference	P
		ref	% difference	P	% difference	P	% difference	P	% difference	P				
cIMT at CCA	Total apoA-I	0%	-0.3%	0.82	1.5%	0.22	-0.1%	0.94	-0.6%	0.59	0.70	0.0%	0.90	
	ApoA-I without apoC-III	0%	-1.5%	0.18	0.4%	0.72	-1.7%	0.17	-3.7%	0.005	0.01	-1.0%	0.02	
	ApoA-I with apoC-III	0%	0.6%	0.60	2.9%	0.02	2.8%	0.02	4.4%	0.001	0.001	1.5%	0.001	
cIMT max	Total apoA-I	0%	-1.0%	0.63	2.2%	0.32	1.0%	0.64	-1.9%	0.40	0.58	-0.5%	0.49	
	ApoA-I without apoC-III	0%	-1.0%	0.64	-0.7%	0.75	-2.1%	0.36	-7.3%	0.003	0.003	-2.1%	0.02	
	ApoA-I with apoC-III	0%	-0.6%	0.79	6.3%	0.01	3.1%	0.19	7.9%	0.003	0.002	2.2%	0.01	
composite cIMT	Total apoA-I	0%	-0.8%	0.63	1.9%	0.26	0.5%	0.76	-1.4%	0.39	0.56	-0.3%	0.52	
	ApoA-I without apoC-III	0%	-1.2%	0.47	-0.4%	0.82	-2.0%	0.25	-6.0%	0.001	0.002	-1.7%	0.009	
	ApoA-I with apoC-III	0%	-0.2%	0.89	5.0%	0.004	2.9%	0.10	6.5%	0.001	0.0004	1.9%	0.003	

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI. Analysis of apoA-I without and with apoC-III simultaneously adjusted.

Table 3-3. Association between apoC-III concentrations and cIMT

Outcome	Exposure	Quintiles									P-trend	per-SD	
		Q1	Q2		Q3		Q4		Q5				
		ref	% difference	p	% difference	p	% difference	p	% difference	p		% difference	p
cIMT at CCA	Total apoC-III	0%	0.6%	0.60	1.0%	0.39	2.4%	0.04	2.2%	0.06	0.02	1.2%	0.002
	ApoC-III in HDL	0%	1.4%	0.22	2.1%	0.07	3.2%	0.01	4.6%	0.0003	0.0003	1.6%	0.0001
	ApoC-III to apoA-I ratio	0%	3.1%	0.01	2.7%	0.02	2.7%	0.03	2.7%	0.03	0.10	0.6%	0.11
cIMT max	Total apoC-III	0%	2.2%	0.33	3.4%	0.13	5.1%	0.02	3.4%	0.14	0.10	1.1%	0.13
	ApoC-III in HDL	0%	1.0%	0.66	3.4%	0.14	5.4%	0.02	5.9%	0.02	0.007	1.6%	0.03
	ApoC-III to apoA-I ratio	0%	3.0%	0.18	4.3%	0.07	0.6%	0.78	1.5%	0.54	0.99	0.5%	0.48
composite cIMT	Total apoC-III	0%	1.4%	0.39	2.4%	0.14	4.1%	0.02	2.9%	0.08	0.045	1.1%	0.04
	ApoC-III in HDL	0%	1.1%	0.51	2.9%	0.09	4.5%	0.01	5.4%	0.003	0.001	1.6%	0.005
	ApoC-III to apoA-I ratio	0%	2.9%	0.08	3.7%	0.03	1.4%	0.40	1.9%	0.28	0.66	0.6%	0.31

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI.

Table 3-4. Association between apoB concentrations and cIMT

Outcome	Exposure	Quintiles										P-trend	per-SD	
		Q1	Q2		Q3		Q4		Q5		p		% difference	p
		ref	% difference	p	% difference	p	% difference	p	% difference	p				
cIMT at CCA	Total apoB	0%	1.2%	0.32	2.1%	0.07	2.6%	0.03	3.3%	0.01	0.004	1.4%	0.0003	
	ApoB without apoC-III	0%	1.8%	0.13	1.6%	0.21	1.9%	0.13	2.7%	0.06	0.07	1.3%	0.004	
	ApoB with apoC-III	0%	-1.6%	0.18	-0.9%	0.44	1.8%	0.16	0.1%	0.96	0.37	0.1%	0.76	
cIMT max	Total apoB	0%	1.7%	0.45	2.8%	0.21	3.6%	0.12	4.3%	0.07	0.047	1.7%	0.02	
	ApoB without apoC-III	0%	-0.7%	0.74	2.7%	0.26	1.9%	0.44	0.5%	0.85	0.60	1.1%	0.19	
	ApoB with apoC-III	0%	-1.9%	0.40	1.1%	0.64	3.1%	0.21	2.1%	0.42	0.18	1.0%	0.23	
composite cIMT	Total apoB	0%	1.4%	0.40	2.4%	0.14	3.2%	0.06	4.0%	0.02	0.01	1.6%	0.004	
	ApoB without apoC-III	0%	0.1%	0.95	2.2%	0.22	1.9%	0.30	1.3%	0.51	0.34	1.2%	0.06	
	ApoB with apoC-III	0%	-1.9%	0.26	0.2%	0.89	2.6%	0.15	1.3%	0.52	0.18	0.7%	0.29	

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI. Analysis of apoB without and with apoC-III simultaneously adjusted.

Supplementary Table 3-1. Association between apoA-I concentrations and cIMT after adjusting for TG

Outcome	Exposure	Quintiles										P-trend	per-SD	
		Q1	Q2		Q3		Q4		Q5		P		% difference	P
		ref	% difference	P	% difference	P	% difference	P	% difference	P				
cIMT at CCA	Total apoA-I	0%	-0.3%	0.77	1.8%	0.12	-0.1%	0.94	-0.5%	0.66	0.77	0.0%	0.94	
	ApoA-I without apoC-III	0%	-1.3%	0.25	0.8%	0.49	-1.2%	0.35	-3.0%	0.03	0.06	-0.8%	0.11	
	ApoA-I with apoC-III	0%	0.3%	0.80	2.3%	0.06	2.2%	0.08	3.2%	0.03	0.02	1.1%	0.03	
cIMT max	Total apoA-I	0%	-1.1%	0.62	2.4%	0.28	1.1%	0.62	-1.8%	0.42	0.60	-0.5%	0.49	
	ApoA-I without apoC-III	0%	-1.0%	0.63	-0.8%	0.72	-2.1%	0.38	-7.4%	0.003	0.004	-2.1%	0.02	
	ApoA-I with apoC-III	0%	-0.5%	0.81	6.3%	0.009	3.2%	0.18	7.9%	0.005	0.003	2.2%	0.02	
composite cIMT	Total apoA-I	0%	-0.8%	0.61	2.1%	0.20	0.6%	0.74	-1.3%	0.42	0.59	-0.3%	0.52	
	ApoA-I without apoC-III	0%	-1.1%	0.50	-0.3%	0.86	-1.8%	0.31	-5.8%	0.002	0.004	-1.6%	0.02	
	ApoA-I with apoC-III	0%	-0.3%	0.84	4.8%	0.007	2.8%	0.12	6.1%	0.003	0.002	1.8%	0.01	

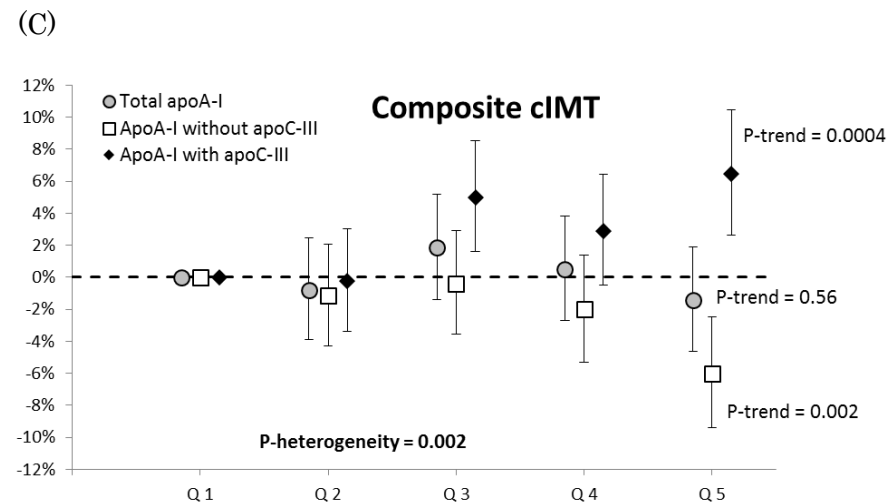
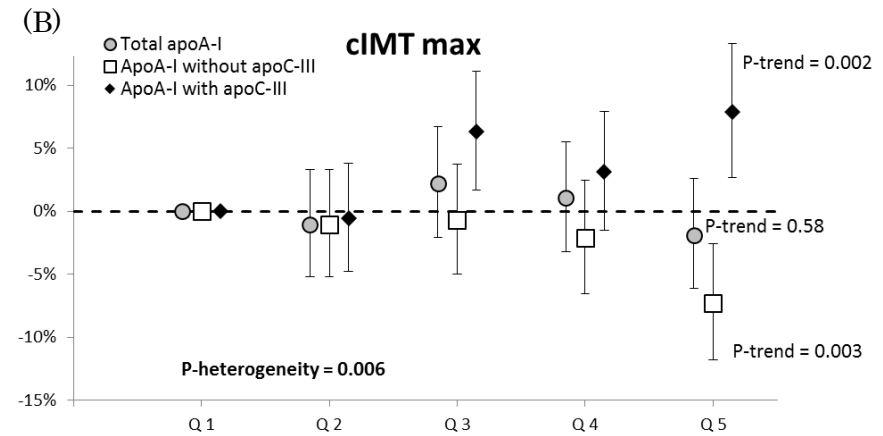
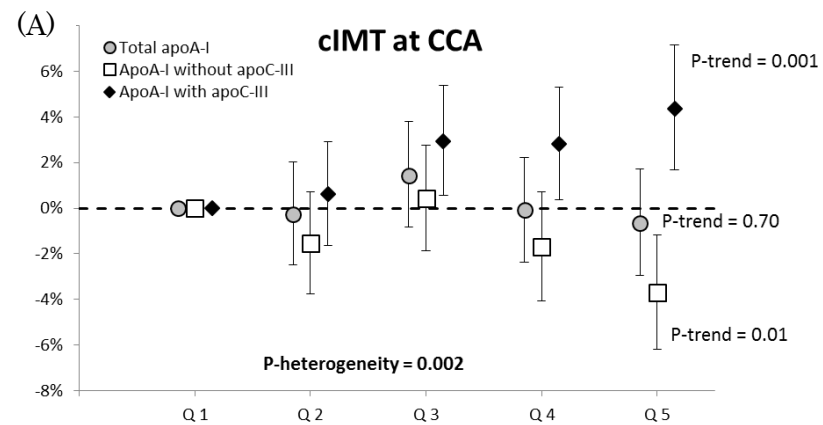
Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity, BMI and TG. Analysis of apoA-I without and with apoC-III simultaneously adjusted.

Supplementary Table 3-2. Association between apoC-III concentrations and cIMT after adjusting for TG

Outcome	Exposure	Quintiles									P-trend	z-score	
		Q1	Q2	Q3		Q4		Q5					
		ref	% difference	p	% difference	p	% difference	p	% difference	p	p	% difference	p
cIMT at CCA	Total apoC-III	0%	0.3%	0.79	0.4%	0.76	1.7%	0.17	0.3%	0.82	0.58	0.7%	0.14
	ApoC-III in HDL	0%	1.2%	0.31	1.7%	0.16	2.6%	0.05	3.3%	0.03	0.03	1.3%	0.02
	ApoC-III to apoA-I ratio	0%	2.7%	0.02	2.3%	0.06	1.9%	0.13	1.2%	0.40	0.76	0.0%	0.94
cIMT max	Total apoC-III	0%	1.9%	0.38	3.0%	0.18	4.9%	0.04	2.5%	0.35	0.23	0.9%	0.32
	ApoC-III in HDL	0%	1.2%	0.61	3.7%	0.11	5.9%	0.02	6.6%	0.03	0.009	2.1%	0.05
	ApoC-III to apoA-I ratio	0%	2.7%	0.23	4.0%	0.09	0.0%	0.99	0.1%	0.96	0.58	0.2%	0.83
composite cIMT	Total apoC-III	0%	1.2%	0.48	1.9%	0.25	3.6%	0.04	1.7%	0.40	0.24	0.8%	0.21
	ApoC-III in HDL	0%	1.1%	0.50	2.9%	0.09	4.6%	0.01	5.4%	0.01	0.005	1.8%	0.02
	ApoC-III to apoA-I ratio	0%	2.7%	0.12	3.4%	0.05	0.7%	0.68	0.5%	0.79	0.72	0.1%	0.84

Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity, BMI and TG.

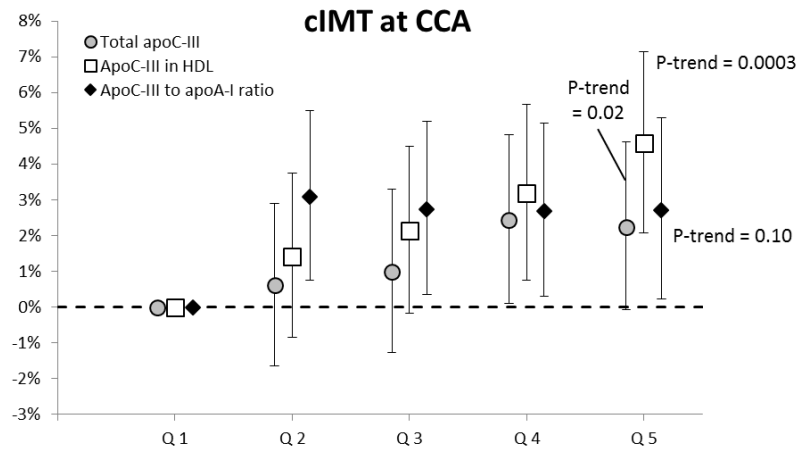
Figure 3-1. Association between apoA-I concentrations and cIMT



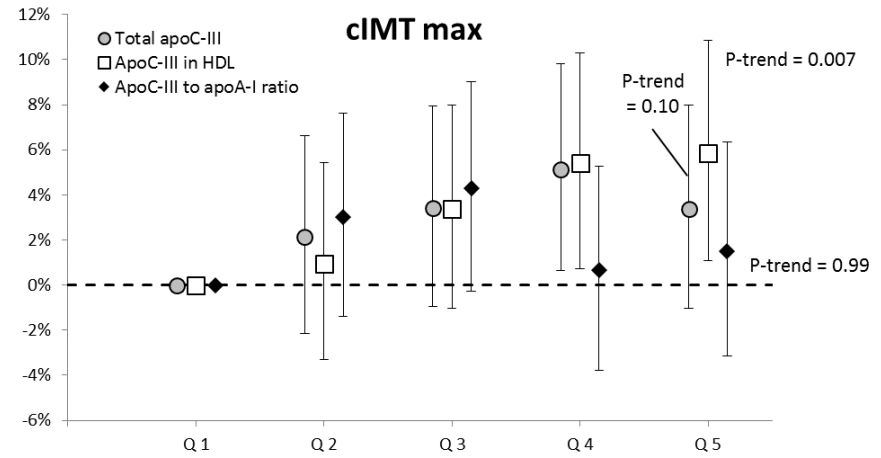
Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI. Analysis of apoA-I without and with apoC-III simultaneously adjusted. P-heterogeneity in panels comparing trends across quintiles of apoA-I with and without apoC-III.

Figure 3-2. Association between apoC-III concentrations and cIMT

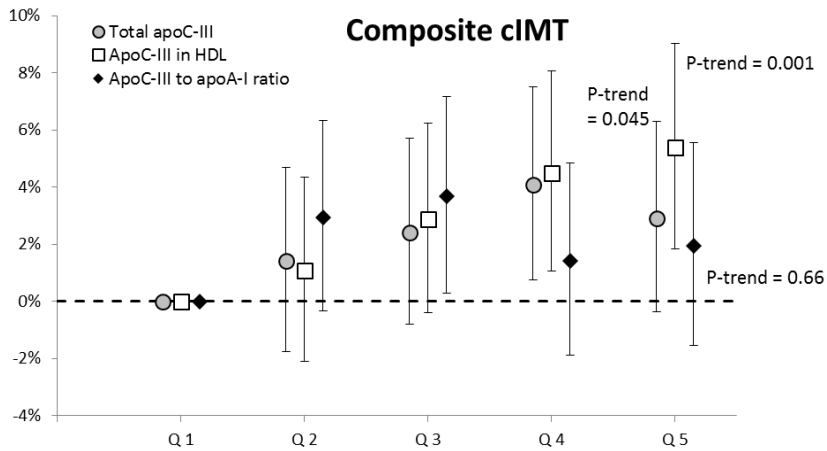
(A)



(B)



(C)



Multivariable linear regression models adjusted for age, sex, center, alcohol, smoking, physical activity and BMI.

